Medullary Astrocytes May Modulate the Depressed Excitability of Rat Face Primary Motor Cortex (Face-M1) Induced by Noxious Tooth Pulp Stimulation

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Faculty of Dentistry
University of Toronto

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Abstract

Acute inflammatory dental pain is a prevalent pain condition that is often associated with disruption of orofacial sensorimotor functions. The aims of this project were to examine whether application of the inflammatory irritant mustard oil (MO) to the rat maxillary molar tooth pulp affects face-M1 excitability manifested as a change in intracortical microstimulation threshold intensity for evoking jaw-opening muscle electromyographic activity, and if subsequent application of the astrocytic inhibitor methionine sulfoximine (MSO) to the medullary dorsal horn (MDH) can modulate the MO-induced effects. Compared with vehicle, pulpal MO application significantly decreased face-M1 excitability. Subsequent MSO application to the MDH attenuated the MO-induced effects. These novel findings suggest that acute noxious stimulation of the dental pulp is associated with decreased face-M1 excitability that may be, in part, dependent on the functional integrity of MDH astrocytes. Therefore, cortical and subcortical mechanisms may contribute to limit jaw movements in acute inflammatory dental pain.
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<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>Alternating current</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ACC</td>
<td>Anterior Cingulate Cortex</td>
</tr>
<tr>
<td>AD</td>
<td>Anterior digastric</td>
</tr>
<tr>
<td>AG</td>
<td>Agranular</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic</td>
</tr>
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<td>AMPARS</td>
<td>AMPA receptors</td>
</tr>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AP</td>
<td>Anteroposterior</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CAN</td>
<td>Canada</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic-adenosine-5’,3’-monophosphate</td>
</tr>
<tr>
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<td>Caspase-6</td>
</tr>
<tr>
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<td>Chemokine (C-C motif) ligand 21</td>
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<td>CD-14</td>
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<tr>
<td>CFA</td>
<td>Complete Freund’s Adjuvant</td>
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<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
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<td>CGRPR</td>
<td>CGRP receptor</td>
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<td>Central nervous system</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
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<td>CX30</td>
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<td>DC</td>
<td>Direct Current</td>
</tr>
<tr>
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<td>Deoxyribonucleic acid</td>
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<tr>
<td>DNIC</td>
<td>Diffuse noxious inhibitory controls</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyographic</td>
</tr>
<tr>
<td>ERK1</td>
<td>Extracellular signal-regulated kinase type 1</td>
</tr>
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</tr>
<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potentials</td>
</tr>
<tr>
<td>FA</td>
<td>Fluoroacetate</td>
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<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>G</td>
<td>Granular</td>
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<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<td>GG</td>
<td>Genioglossus</td>
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<tr>
<td>GluR1</td>
<td>Glutamate receptor type 1 subunit</td>
</tr>
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<td>GluR2</td>
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</tr>
<tr>
<td>GluR3</td>
<td>Glutamate receptor type 3 subunit</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine Synthetase</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrogen Chloride</td>
</tr>
<tr>
<td>HPC</td>
<td>Polymodal heat, pinch or cold cells</td>
</tr>
<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
</tr>
<tr>
<td>Iba1</td>
<td>Ionized calcium-binding adapter molecule 1</td>
</tr>
<tr>
<td>ICMS</td>
<td>Intracortical microstimulation</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
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</tr>
<tr>
<td>ILB4</td>
<td>Isolectin B4</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>IR</td>
<td>Immunoreactivity</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
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</table>
JNK c-Jun N-terminal kinase
KA Kainate
LAD Left anterior digastric muscle
LPS Lipopolysaccharide
LTM Low-threshold mechanoreceptive
M1 Primary motor cortex
MAPK Mitogen-activated protein kinase
MDH Medullary dorsal horn
MeAIB Methylamino-isobutyric acid
MEPs Motor evoked potentials
Mg$^{2+}$ Magnesium ion
mGluR Metabotropic glutamate receptor
MHC II Major histocompatibility complex) class II
MIN Mineral oil
MK801 Dizocilpine
ML Mediolateral
MO Mustard oil
MRS Magnetic resonance spectroscopy
MSO Methionine sulfoximine
NK1 Neurokinin-1
NMDA N-methyl-D-aspartate
NMDAR NMDA receptor
NR1 NMDAR subtype 1 subunit
NR2A-D NMDAR subtype 2 subunit
NR3A/B NMDAR subtype 3 subunit
NS Nociceptive specific
P2X4 Purinoceptor subtype 2X4
p38 MAPK P38 mitogen-activated protein kinases
PAG Periaqueductal gray
PBS Phosphate-buffered saline
PDE IV Phosphodiesterase
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PSD</td>
<td>Postsynaptic density zone</td>
</tr>
<tr>
<td>RAD</td>
<td>Right anterior digastric</td>
</tr>
<tr>
<td>RF</td>
<td>Receptive field</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>S1</td>
<td>Primary somatosensory cortex</td>
</tr>
<tr>
<td>S2</td>
<td>Secondary somatosensory cortex</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SG</td>
<td>Substantia gelatinosa</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMS</td>
<td>Transcranial magnetic stimulation</td>
</tr>
<tr>
<td>TNF-$\alpha$</td>
<td>Tumor necrosis factor $\alpha$</td>
</tr>
<tr>
<td>Trkb</td>
<td>Tyrosine receptor kinase type b</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential family of channels</td>
</tr>
<tr>
<td>TRPA1</td>
<td>Transient receptor potential ankyrin 1</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanilloid 1</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>VBSNC</td>
<td>Trigeminal brainstem somatosensory complex</td>
</tr>
<tr>
<td>Vc</td>
<td>Subnucleus caudalis</td>
</tr>
<tr>
<td>Vi</td>
<td>Subnucleus interpolaris</td>
</tr>
<tr>
<td>VL</td>
<td>Ventral lateral nucleus</td>
</tr>
<tr>
<td>Vm</td>
<td>Trigeminal motor nucleus</td>
</tr>
<tr>
<td>Vo</td>
<td>Subnucleus oralis</td>
</tr>
<tr>
<td>VP</td>
<td>Ventroposterior nucleus</td>
</tr>
<tr>
<td>VPL</td>
<td>Ventral posterolateral nucleus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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</tr>
<tr>
<td>VPM</td>
<td>Ventroposteriomedial nucleus</td>
</tr>
<tr>
<td>Vsp</td>
<td>Spinal tract nucleus</td>
</tr>
<tr>
<td>WDR</td>
<td>Wide-dynamic range</td>
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Chapter 1
Introduction

Dental inflammatory pain is one of the most common reasons patients seek dental or medical treatment, and is often associated with disruption of orofacial musculoskeletal functions. This thesis will review the literature showing that application of the inflammatory irritant mustard oil (MO) to the dental pulp in rats has been widely used to study brainstem mechanisms associated with pulpal inflammation and pain. These studies have shown that pulpal MO application produces a glutamate receptor-dependent increased neuronal excitability (central sensitization) of medullary dorsal horn (MDH) nociceptive neurones reflected in increased mechanoreceptive field size, orofacial-evoked responses and decreased activation thresholds. This MDH central sensitization has also been associated with increased immunoreactivity (IR) of MDH non-neuronal astrocyte cells and both of these MO-induced changes can be prevented or reversed by MDH application of astrocyte inhibitors including L-methionine sulfoximine (MSO), a specific inhibitor of the astrocyte glutamate-glutamine shuttle that converts glutamate to glutamine. These findings suggest that maintenance of MDH central sensitization induced by pulpal MO application is dependent on replenishment of glutamate by astrocytes.

Second-order neurones within the MDH project mainly via the thalamus to the face primary somatosensory cortex (face S1), the main cortical somatosensory receptive area for the orofacial region. However, the face primary motor cortex (face-M1), the main cortical region crucial for the initiation and control of orofacial movements, also receives considerable somatosensory inputs (including nociception) from the teeth and other orofacial tissues either indirectly through the face-S1 or directly through the thalamus. This sensory input from the orofacial environment provides peripheral somatosensory information that plays a crucial role in modulating face-M1 motor output and subsequently orofacial motor function. Experimentally induced orofacial pain is associated in animals and humans with decreased face-M1 excitability manifested as increased intracortical microstimulation (ICMS) or transcranial magnetic stimulation (TMS) thresholds for evoking electromyographic (EMG) activity in orofacial muscles. However, it is unclear whether acute dental pain induced by MO application to the rat tooth pulp is also associated with decreased face-M1 excitability.
and whether these changes involve MDH astrocyte function. The subsequent sections of this thesis will briefly review the topic of pain and how dental pulp innervation and central nervous system (CNS) processing of dental pulp sensory information are suited for the study of pain. Central pathways of sensation will then be detailed to explain how pain can affect motor function, and the technique of ICMS will then be considered as a method to examine central mechanisms of motor function. The hypothesis and objectives of this thesis will then be stated, followed in sequence by an outline of the methodological approaches used to address those objectives, the results obtained, and a general discussion of the findings and their implications.

1. Pain

Pain is defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (IASP Taxonomy, 2011; Tracey and Mantyh, 2007). Pain can be categorized by the duration of pain or the cause of pain. Pain lasting for a short duration of hours or days is termed acute pain, while pain lasting for more than 3 months is termed chronic pain (Katz, 1998; Sessle, 2009b; Sessle, 2000; Tracey and Mantyh, 2007). Acute pain can result from many sources including damage to nerves (i.e. neuropathic pain), from inflammation after injury to peripheral tissue (i.e. inflammatory pain), or as a result of a systemic disease such as cancer (Lam et al., 2012; Lynch, 2011; Portenoy and Lesage, 1999; Renn and Dorsey, 2005; Katz, 1998; Wall and Melzack, 1999).

1.1. Epidemiology of acute dental pain

Acute pain was the primary cause for seeking emergency care in 70% of patients in a survey of hospitals in the United States (US) and Canada (CAN), and loss of productivity due to acute and chronic pain conditions (headache, backpain, arthritis, musculoskeletal) was estimated at $61.2 billion (Cordell et al., 2002; Stewart et al., 2003). In regards to oral pain, the Canadian Health Measures Survey from 2007-2009 showed 11.7% of respondents reported oral pain in the past 12 months (Statistics Canada), while a survey in the US reported almost half of respondents (44.3%) had more than 5 toothaches in 10 years (Cohen...
Reports of dental pain prevalence in Canada are lacking, although a survey in Toronto in 1985 showed 14.1% of adults reported a “toothache” in the past month (Locker and Grushka, 1987). Therefore, acute dental pain is still a clinical challenge, and novel treatment options are an ongoing area of research.

1.2. Pathophysiology and clinical manifestations of acute dental pain

The most common cause of dental pain is caries. Lesions arising from caries can first penetrate the dentine to induce dentinal pain, causing increased sensitivity to thermal stimuli and sweet substances (Cummins, 2009; Napenas, 2013; Wall and Melzack, 1999). As the carious lesion penetrates deeper into the tooth and reaches the tooth pulp, pulpal pain can be induced by inflammatory processes that result in acute pain following mechanical and thermal stimulation through chewing, touch and temperature (Cummins, 2009; Napenas, 2013; Wall and Melzack, 1999). Bacteria, including acidogenic microorganisms such as Streptococcus mutans, are important for the development of caries (Hahn and Liewehr, 2007; Selwitz et al., 2007). By fermenting dietary carbohydrates, bacteria demineralize enamel and dentine, allowing the lesion to penetrate deeper into the dentin. As the lesion transitions deeper into the dentine, there is a shift from facultative bacteria, like streptococci, to lactobacilli or anaerobic bacteria due to a change in environmental conditions, with altered nutrients and oxygen levels (Hahn and Liewehr, 2007). Once the bacteria penetrate past the dentine to the pulp, immune responses by the host lead to pulpal inflammation and pain or hypersensitivity. These immune responses may be induced by diffusion of bacteria cell wall or metabolic wastes, such as lactic acid, through dentinal tubules (Hahn and Liewehr, 2007). Lipopolysaccharides (LPS), which are endotoxins released from the cell walls of Gram-negative bacteria, are associated with heat sensitivity and pain by activating the Hageman factor which initiates bradykinin (BK) production that sensitizes nociceptors (Khabbaz et al., 2000). LPS can induce release of proinflammatory cytokines such as tumour necrosis factor α (TNF-α), interleukin-1 (IL-1) and interleukin-8 (IL-8), interleukin-12 (IL-12) and anti-inflammatory cytokine interleukin-10 (IL-10) through binding to cluster differentiation 14 (CD14) and Toll-like receptors (TLRs) on macrophages (Hahn and Liewehr, 2007). Bacteria
have also been shown to induce acute pain independent of inflammatory responses, through release of bacterial-derived factors such as N-formylated peptides and α-haemolysin to directly activate tooth pulp afferents (Chiu et al., 2013).

2. Tooth anatomy, orofacial afferents and processes of acute dental pain

2.1 Peripheral nociceptive pathways and processes

2.1.1 Tooth anatomy

The tooth pulp is predominantly innervated by nociceptive fibres, of which 70-90% are C-fibres. Most of the myelinated pulpal axons are Aδ-fibres, though a few are either Aβ-size or autonomic efferent fibres (Bueltman et al., 1972; Fried et al., 2011; Fried and Hildebrand, 1981a,b; Jain et al., 2013). The Aβ-fibres innervating the pulp and periodontal tissues of the tooth mediate dental touch and proprioception, while the Aδ and C afferent fibres mediate nociception. Rat molar tooth pulps receive innervation from nearly 230 myelinated fibres and 800 unmyelinated fibres, compared to the cutaneous innervation of the lower limb where small fibre C and Aδ density has been measured at 21 fibres/mm (Paxinos, 2004; McArthur et al., 1998). The tooth pulp functions primarily as a nociceptive sensor, but also contains support cells such as odontoblasts and fibreblasts to maintain and repair dentin. It is protected from the oral environment by surrounding cementum, dentine and enamel.

The inner part of the dentine is innervated by pulpal axons, and a single pulpal axon may be associated with 100 dentinal tubules, though they only extend up to 100-150 µm into the tubules (Byers, 1985). Deeper into the tooth at the coronal level, the odontoblast region is also innervated by unmyelinated pulpal axons, creating the marginal plexus (or the subodontoblastic plexus of Raschkow) at the pulpal dentinal border. Pulpal axons then travel through the root of the tooth through the apical foramen and accessory foramina, and bundle into fascicles to exit through the root canal next to blood vessels (Fig. 1-1). Multiple tooth pulps may be innervated by the same trigeminal primary afferent neurone. In rats and other mammals, the pulpal axons from the maxillary teeth converge into the maxillary nerve, while
pulpal axons from the mandibular teeth converge into the mandibular nerve (specifically its inferior alveolar branch); both are divisions of the trigeminal nerve (Paxinos, 2004).

Dental pulp sympathetic axons wrap around blood vessels in the pulp to regulate blood flow but also can regulate the excitability of dental afferents (see Sessle, 1987; Jain et al., 2013). However, innervation by sympathetic nerves is limited, as sympathectomy does not drastically reduce the number of pulpal axons (Fried and Hildebrand, 1978; Noga and Holland, 1983). Autonomic neurones have their cell bodies in the superior cervical ganglion.

Fig.1-1. Drawing of molar tooth anatomy. (www.graysanatomyonline.com)
2.1.2 Dental pain processes

Acute dental pain can manifest clinically as a fast-onset, short-duration, sharp pain when A-δ fibres are stimulated through a hydrodynamic process (Abd-Elmeguid and Yu, 2009; Chung et al., 2013; Jain et al., 2013). The hydrodynamic effect occurs as dentinal fluid in the dentinal tubules is displaced in response to dentinal stimulation (e.g., drilling, sweet foods, cold air, and hypertonic solutions); rapid flow of dentinal fluid can then activate intra-dental A-δ nerve fibres and pulpal nociceptors (Abd-Elmeguid and Yu, 2009; Chung et al., 2013; Cummins 2009; Jain et al., 2013; Narhi et al., 1982). A slow-onset, dull pain is manifested clinically when C-fibres are stimulated, which respond primarily to heat. Although C-fibres also respond to cooling, cold stimuli only induce the rapid pain characteristic of A-δ fibre activation. On prolonged cooling, pulp vessels begin constricting and can cause anoxia, which prevents A-δ/C fibre activation and decreases impulse frequency/excitability (Bender, 2000; Jain et al., 2013). Due to the deeper location of C-fibres in the core or central region of the pulp, C-fibre pain signals irreversible pulpitis (Jain et al., 2013).

2.1.3 Orofacial primary afferents: Aβ, Aδ and C fibres

Aβ-fibres are large diameter, thickly myelinated afferents that are fast-conducting, mediate innocuous sensations such as touch and vibration, and have specialized terminations that respond to mechanical stimuli or movement (Sessle, 2000, 2009a). Nociceptive and thermoreceptive A-δ and C-fibres are small-diameter, thinly myelinated, or non-myelinated in the case of C-fibres, and so are slowly conducting sensory afferents that terminate in the periphery as free nerve endings (Sessle, 2000, 2006). C-fibres are classified into two groups, peptidergic and nonpeptidergic. Peptidergic fibres contain neuropeptides like substance P (SP) and are associated with deeper regions of the skin and other tissues, while nonpeptidergic C-fibres primarily innervate the epidermis (Lawson et al., 1997; Snider et al., 1998; Todd, 2010).
2.1.4 Activation of nociceptive afferents by inflammatory irritants

Inflammatory irritants used to evoke acute experimental pain are numerous, and their application to orofacial tissues mimics the action of local inflammatory mediators released by tissues following injury (Sessle, 2011a; Bautista et al., 2013). Capsaicin, MO, formalin, zymosan, Complete Freud’s Adjuvant (CFA) are all inflammatory irritants that can induce acute pain through activation of various receptors on nociceptive afferents, including the family of transient receptor potential (TRP) channels and TLRs (Chiang et al., 2012; Sessle, 2011b).

2.1.5 Mustard oil and TRPA1 receptors

MO is a natural substance that can activate the transient receptor potential ankyrin 1 (TRPA1) receptor (Eid and Cortright, 2009). As a channel found in the TRP family, TRPA1 can also be activated by other exogenous ligands (e.g., cinnamaldehyde), heat, protein kinase activity, phospholipids, and osmolarity or pH changes (Eid and Cortright, 2009). Since TRPA1 receptors are expressed in a subset of C-fibres, MO can induce acute pain, hyperalgesia and neurogenic inflammation in animals and humans by binding to TRPA1 receptors to activate C-fibres (Basbaum et al., 2009; Bautista, 2013). TRPA1 can be opened by calcium ($Ca^{2+}$) influx through transient receptor potential vanilloid 1 (TRPV1) channels, which are phosphorylated after BK receptor activation induces second messenger signaling by phospholipase C (PLC) (see Fig. 1-2) (Bautista et al., 2006), allowing for MO to be used experimentally to activate C-fibres and induce inflammatory pain (Bautista, 2013; Jordt et al., 2004).
Inflammation. A significant factor in pulpal inflammation is the sensitization of intradental afferents. Released after soft tissue stimuli, decreased activation responses are no longer correlated in TRPV1-deficient neurons, respectively. Additionally, PLC activation does not robustly activate TRPV1, and TRPA1 resembles canonical receptor-operated TRP channels in that activation of the PLC pathway produces rapidly developing inward currents, even in the absence of other known stimuli. TRPA1 has therefore been proposed as a likely candidate to mediate the acute excitatory response. This is further supported by our observations that the magnitudes of MO- and bradykinin-evoked responses are no longer correlated in TRPV1-deficient mice. Moreover, the haploinsufficiency phenotype that we observed (i.e., decreased activation thresholds, and spontaneous activity (Chiang et al., 2012; Sessle, 2011b). Cytokines and other inflammatory mediators (see below) can diffuse through the tissue and activate adjacent nociceptors. The same inflammatory mediators released after soft tissue injury are released after pulpal injury or inflammation and can sensitize intradental afferents.

The activation of pulp tissue cells and vasodilation of blood vessels contributes to pulpal inflammation. A significant factor in pulpal inflammation is the activation of C-fibres, which

**Fig. 1-2.** Activation of TRPA1 channels by MO requires the concerted action of Ca\(^{2+}\) through intracellular Ca\(^{2+}\) stores and TRPV1 receptors, which are phosphorylated by enzymes downstream of BK receptor activation. (Abbreviations: BK – bradykinin; Ca\(^{2+}\) – calcium ion; IP\(_3\) – inositol triphosphate; PIP\(_2\) - phospatidylinositol 4,5-bisphosphate; PKC – protein kinase C; PLC – phospholipase C; MO – mustard oil; TRPA1 – transient potential receptor ankyrin 1; TRPV1 – transient potential receptor vanilloid 1. Adapted from Bautista et al., 2006.)

**2.1.6 Peripheral sensitization**

An important functional feature of nociceptive primary afferents is their ability to become sensitized. Sensitization of nociceptive primary afferents can occur as a result of nerve injury or inflammation and is associated with increased excitability or sensitization to subsequent stimuli. After injury or inflammation, peripheral nociceptors show increased responses to noxious stimuli, decreased activation thresholds, and spontaneous activity (Chiang et al., 2012; Sessle, 2011b). Cytokines and other inflammatory mediators (see below) can diffuse through the tissue and activate adjacent nociceptors. The same inflammatory mediators released after soft tissue injury are released after pulpal injury or inflammation and can sensitize intradental afferents.
mediate pain signals and can be activated by noxious thermal, mechanical and chemical stimuli. C-fibres can also release neurokinin substance P (SP), neurokinin A and B and calcitonin gene-related peptide (CGRP) (Bautista et al., 2013; Hildebrand et al., 1995; Jain et al., 2013). SP released by depolarization of C-fibres results in activation of immune cells including lymphocytes, granulocytes and macrophages by acting on their neurokinin 1 (NK1) receptors (Bautista et al., 2013; Jain et al., 2013). Pulp tissue cells have been shown to release mediators including BK, prostaglandins, leukotrienes, histamine, TNF-α, IL-1, serotonin, opioids and cytokines (Jain et al., 2013; Sessle, 2006). This “inflammatory soup” can increase nociceptive afferent excitability and induce release of more SP and CGRP from C-fibres, further prolonging the pulpal inflammation (Lembeck and Holzer, 1979; Louis et al., 1989; Ren and Dubner, 2010; Sessle 2011a). Nevertheless, some of these mediators (e.g. opioids) may suppress pain (Dionne et al., 2001; Jaber et al., 2003). These peripheral mechanisms can explain the clinical manifestation of primary hyperalgesia, allodynia, spontaneous pain and pain spread, that are associated with acute (and chronic pain) conditions. Allodynia refers to pain induced by normally innocuous stimuli, while primary hyperalgesia refers to an increased pain response due to noxious stimulation (Latremoliere and Woolf, 2009; Sessle, 2011b).

### 2.2. Central nociceptive pathways and processes

#### 2.2.1 Trigeminal somatosensory pathways

Orofacial afferents supplying orofacial tissues including the skin, mucosa, periodontal tissues, and tooth enter the brainstem at the pontine level, synapsing onto the trigeminal brainstem sensory complex (VBSNC) (Dostrovsky, 2006; Sessle, 2009a; Sessle, 2006). The VBSNC is comprised of the main sensory nucleus and the spinal tract nucleus (Vsp), which is further divided into the subnucleus oralis (Vo), subnucleus interpolaris (Vi) and subnucleus caudalis (Vc). (Sessle, 2009). Nociceptive afferents project to the ipsilateral and contralateral VBSNC, but most nociceptive afferents terminate in layers I and V of the Vc (Sessle, 2009; van Steenberghe and De Laat, 1989). Second-order brainstem sensory neurones project contralaterally, as well as ipsilaterally, to the ventral posterior medial nucleus (VPM) of the posterior thalamus and to the posterior nucleus (Po) in rats, to the
VPM, Po, caudal ventral posterolateral nucleus (VPL) and the ventral and caudal portion of the medial dorsal nucleus (MDvc) in monkeys, and ventroposterior nucleus (VP) in humans (Rats: Chiaia et al., 1991; Diamond et al., 1992; Monkeys: Craig, 2004; Graziano and Jones, 2004; Jones et al., 1986; Humans: Dostrovsky, 2006; Nash et al., 2010). Thalamic neurones in the VPM project mainly to the primary somatosensory cortex (S1), which is the main cortical region for the spatiotemporal coding of touch and pain. Although the face-M1 receives sensory information directly from the thalamic motor nuclei (ventral lateral nucleus, VL), it also receives sensory information from axon collaterals and interneurons from the face-S1 through intracortical connections (Keller et al., 1996; Schwark and Jones, 1989; Sessle, 2011a). The thalamic nuclei also target other cortical areas, including the insula, secondary somatosensory cortex (S2) and the anterior cingulate cortex (ACC) to modulate the affective-motivational aspect of pain (Dostrovsky and Sessle, 2005, 2007, 2009, 2011a).

2.2.2 Trigeminal brainstem sensory nuclear complex

Jaw muscle spindle afferents and mechanosensitive afferents of periodontal tissues have their cell bodies in the trigeminal mesencephalic nucleus, which is the rostral component of the VBSNC (Dostrovsky, 2006; Sessle, 2006, 2009a). Projection neurones from the mesencephalic nucleus synapse on the V motor nucleus (Vm) and interneurones in the adjacent supratrigeminal nucleus and Vo to modulate orofacial reflexes (Dostrovsky, 2006; Sessle, 2006, 2009a). The Vc also receives projections from other cranial nerves (e.g. VII, IX, X and XII) (Dostrovsky, 2006; Sessle, 2006, 2009a). The Vc merges caudally with the spinal dorsal horn, and due to its structural and functional similarities with the latter, the Vc is often referred to as the medullary dorsal horn (MDH). Structurally, it is separated into characteristic laminae, with laminae I named the marginal laminae and laminae II the substantia gelatinosa (SG), which contains interneurones. Large-diameter afferents from superficial tissues such as skin terminate in the main sensory nucleus and in all three subnuclei of the VBSNC, although in the MDH they terminate primarily in the deeper layers (laminae III/IV). Small-diameter nociceptive and thermosensitive afferents primarily terminate in the superficial layers I and II of the MDH, but they also have projections to the other two subnuclei of the VBSNC as well (Dostrovsky, 2006; Sessle, 2006, 2009a).
The nuclei and subnuclei of the VBSNC display an inversed dorsoventral somatotopy, where ophthalmic afferents terminate ventrally in each nuclei/subnuclei, mandibular afferents terminate dorsally, and maxillary afferents terminate in between. Oral and perioral structures are found medially, although in the Vc perioral regions are represented rostrally and lateral regions of the face are found more caudally (Sessle, 2005, 2006, 2009a).

2.2.3 Neurones of the trigeminal brainstem sensory nuclear complex

The MDH is a major area for nociceptive processing due to the nature of its afferent inputs, which are mainly nociceptive, and the morphology of its neurones, which resemble spinal dorsal horn neurones. The superficial layers of the MDH primarily contain nociceptive-specific (NS) neurones that are crucial for nociceptive processing, but can also respond to innocuous thermal stimuli. The deeper layers of MDH contain low-threshold mechanoreceptive (LTM), wide-dynamic range (WDR) and NS neurones. LTM neurones in the MDH and other parts of the VBSNC receive orofacial tactile stimuli signaling spatiotemporal features. They receive input from large-diameter afferents that may release glutamate, are activated by light tactile stimuli restricted to a localized receptive field (RF), and display altered responsiveness to increased stimulus intensity or area of RF stimulation (Sessle, 2009a). Noxious stimuli can activate either WDR neurones or NS neurones in the superficial and deeper layers. The nociceptive neurones in laminae I of MDH can be subclassified into NS, WDR and polymodal nociceptive heat, pinch or cold (HPC) (Cervero and Jensen, 2006; Craig, 2003). These neurones receive input from Aδ or C fibres. Nociceptive afferents release SP, ATP and glutamate to activate NS and WDR neurones (Sessle, 2011b). NS cells are fusiform (platelet like), WDR and HPC cells are multipolar, and cold cells are pyramidal (Craig, 2003). The NS and WDR neurones of the MDH innervate both deep tissues (e.g. tooth pulp, cerebrovasculature, TMJ and jaw muscle), oral mucosa and skin, and this convergent input is also a factor in central sensitization (Sessle, 2009a). The Vo and Vi have also been implicated in nociceptive processing, as they contain NS and WDR neurones with intraoral and perioral nociceptive RFs (including the tooth pulp), and lesions to the Vo can disturb orofacial nociceptive responses (Dubner and Ren, 2004; Sessle, 2009a). Significantly, tooth pulp stimulation may activate neurones in both rostral and caudal
components of the VBSNC (Sessle 2009). The MDH also is subject to modulatory influences via its laminae II (the SG) that involve NMDA, purinergic and opioid receptor mechanisms (Sessle, 2009a).

### 2.2.4 Projections from the trigeminal brainstem sensory nuclear complex

Projection neurones from the VBSNC and spinal cord can terminate in the thalamus (see below), but also in four different brainstem sites (the reticular formation, catecholamine groups, parabrachial nucleus and periaqueductal gray, PAG) involved in the control of autonomic and muscle reflex responses (Sessle, 2009a; Craig, 2003). They also project to the cerebellum, superior colliculus, PAG, spinal cord or other nuclei in the VBSNC (Dostrovsky, 2006; Sessle, 2006, 2009a). Cells in laminae V and VII of the MDH project to the reticular formation while cells in laminae I project to the other three brainstem sites (Andrew et al., 2003; Yezierski, 1998). The dorsomedial reticular formation receives nociceptive information from laminae I and V, and either descends into the spinal cord or ascends into layer I of the frontal cortex through the ventromedial thalamus to modulate pain (Bernard et al., 1990; Villanueva et al., 1998). Catecholamine groups are important in homeostatic regulation, and the cells in the ventrolateral medulla, nucleus of the solitary tract, the locus coeruleus and the subcoerulear and Kolliker-Fuse regions in the dorsolateral pons receive nociceptive information from the spinal cord and VBSNC (Basbaum and Fields, 1978). They can be autonomic neurones that project to the spinal cord to modulate sympathetic activation, or project to the hypothalamus to regulate other autonomic responses (Loewy and Spyer, 1990). Laminae I neurones of the MDH and spinal cord can also terminate in the parabrachial nucleus, which has neurones that project to the reticular formation, hypothalamus, amygdala, medial thalamus, ventrobasal thalamus, and therefore may be important in autonomic, neuroendocrine and emotional responses to nociceptive activity (Dostrovsky, 2000). Projections from the laminae I of the MDH and spinal cord to the PAG may be important in analgesia, as neurones in the PAG can modulate nociceptive transmission through their descending projections to the rostroventromedial medulla and ascending projections to the hypothalamus and medial thalamus (Dostrovsky, 2006; Mantyh, 1983; Sessle, 2000).
2.2.5 Modulation of trigeminal brainstem sensory nuclear complex

Modulatory processes can either facilitate or inhibit signals from the VBSNC to other components of the VBSNC or higher brain centres. For example, there are deep bundles from the Vc to the Vo that are responsible for modulating processing of pain-related information at the Vo level (Chiang et al., 2002; Sessle, 2009a). As noted before, laminae II of the Vc is the SG, and like the SG in the dorsal horn of the spinal cord, contains inhibitory neurones providing segmental and descending modulation in Vc (Sessle, 2009a). Descending modulation can be elicited from electrical or chemical stimulation of raphe structures (PAG, nucleus raphe magnus, NRM), the rostroventral medulla, the anterior pretectal area, parabrachial area, M1 and the S1 (Chiang et al., 1991, 1994; Sessle, 2000). The brainstem raphe system may be crucial in descending modulation, as inhibiting this area prevents the modulation of nociceptive input induced by cortical and anterior pretectal stimulation (Chiang et al., 1991). These inhibitory processes are believed to be intrinsic mechanisms aiding in analgesia through treatments such as deep brain stimulation and opioid-related drugs (Fields and Basbaum, 1994; Sessle, 1987). Non-noxious stimuli, such as vibratory or tactile, that stimulate large afferent nerve fibres, can also inhibit responses of VBSNC neurones to noxious stimulation of the tooth pulp, temporomandibular joint, and orofacial muscles (Sessle, 1987, 2000; Wall and Melzack, 1994). Stimulation of small fibres may also inhibit nociceptive transmission, in the case of diffuse noxious inhibitory control (DNIC), or facilitate pain signaling, as in the case of central sensitization (see below) (Le Bars et al., 1979; Popescu et al., 2010; Sessle 2011).

The chemical mediators involved in descending and afferent-induced modulation are numerous and varied, and include \( \gamma \)-aminobutyric acid (GABA), serotonin (5-hydroxytryptamine; 5-HT), endogenous opioids, galanin, noradrenaline, muscarine, adenosine and ATP. Agonists and antagonists of these molecules have been found respectively to augment or attenuate the activity of neurones in the VBSNC (Grudt et al., 1995; Salter et al., 1993; Sessle, 2000, 2011; Tokunaga et al., 1992; Travagli 1996;). These molecules may be released by the descending inputs to the VBSNC, or by inhibitory interneurones in the
VBSNC (such as in the SG), while ascending inputs may release chemical mediators at sites involved in descending modulation or afferent-induced (segmental) inhibition (Dubner and Bennett, 1983; Sessle, 1986, 2000).

2.2.6 Thalamic nuclei

The main relay pathway for nociception in the orofacial region to the thalamus is the trigeminothalamic tract. Most VBSNC projection neurones involved in orofacial pain arise from the MDH and terminate in three areas of the posterior thalamus, including the ventrobasal complex (or VP in humans), the Po and the MDvc (Rats: Chiaia et al., 1991; Diamond et al., 1992; Monkeys: Graziano and Jones 2004; Jones et al., 1986; Humans: Nash et al., 2010). In the ventrobasal complex, the VPM is involved in somatosensation of the face and mouth, while the VPL processes limb somatosensory information. The VPM contains neurones similar in response properties to the LTM, NS, WDR and thermosensitive neurones of the VBSNC, and are somatotopically organized to allow for the retention of the RF features of primary and second-order neurones in the orofacial somatosensory pathway. This specificity is not apparent in the Po or medial thalamus. Neurones in the VPM project to the S1, suggesting a role in the spatiotemporal coding of touch and pain. The face-S1 contains LTM neurones that are critical for detection and discrimination of spatiotemporal stimuli applied to the orofacial area. Less numerous are WDR and NS neurones in face-S1, although their existence in face-S1 suggests a role in the sensory-discrimination aspect of pain. The thalamic nuclei also target other cortical areas, including the insula, S2 and ACC to modulate affective and motivational aspect of pain (Sessle, 2005a, 2009a, 2011b; Dostrovsky and Sessle, 2007). This is consistent with brain-imaging studies in humans (Tracey and Mantyh, 2007; Apkarian et al., 2005).

2.2.7 Primary somatosensory cortex

Projections from the VPM to S1 synapse onto layers IV and VI, while Po projects to layer I (Oda et al., 2004). Layer IV of S1 in rats and primates is referred to as the granular cortex as it contains densely packed neurones (Hayama and Ogawa, 1997; Kaas, 1963). In rats, the whiskers are represented in densely packed areas known as “barrels”, while less dense areas are known as septa containing intracortical connections (Kim and Ebner, 1999). Face-S1 is
activated upon stimulation of orofacial tissues, and is organized in a somatotopic manner (Henry et al., 2005; Iyengar et al., 2007; for reviews, see Avivi-Arber et al., 2011; Kaas et al., 1989). This somatotopy of orofacial mechanoreceptive inputs (from teeth, tongue, lips, vibrissae, etc.) is related to the role of face-S1 in localizing and processing orofacial sensory input. This processing also allows S1 to modulate motor activity through its intracortical connections with M1; the term sensorimotor cortex is used to describe the combination of both areas (Asanuma, 1989; Avivi-Arber et al., 2011; Grinevich et al., 2005). M1 is therefore also crucial for sensorimotor integration and control. In regards to pain sensation, opposing reports of a role for S1 in pain processing appear in the literature (Craig, 2003). Although there are projections from VPM to S1, electrical stimulation of S1 rarely elicits pain (Craig, 2003; Ettlin et al., 2004). Imaging and recording studies however have shown activation of S1 in cutaneous pain and tooth pain to suggest it does have a role in pain perception (Apkarian et al., 2005; Iwata et al., 1986, 1987, 1994; Jantsch et al., 2005; Talbot et al., 1991).

2.2.8 Central Sensitization

A congruent nociceptive hypersensitivity to peripheral sensitization occurs in CNS nociceptive neurones of the spinal cord and MDH and is termed central sensitization. Central sensitization is manifested in CNS neurones by increases in membrane excitability, synaptic efficacy or a reduced inhibition. Both central and peripheral sensitization contribute to primary hyperalgesia, allodynia, spontaneous pain and pain spread associated with acute and chronic orofacial pain conditions (Chiang et al., 2012; Sessle, 2011b; Latremoliere and Woolf, 2009). Central sensitization can result in non-nociceptive afferent inputs to the CNS to become nociceptive, as the lowered threshold of CNS nociceptive neurones allows for their depolarization by A β mechanoreceptors. This A β fibre-mediated pain, where stimulation of areas around the initial site of injury can induce pain, is termed secondary hyperalgesia (Latremoliere and Woolf, 2009; Woolf and Salter, 2000). As central sensitization results from excitability changes in CNS nociceptive neurones, peripheral nociceptive inputs may not be required to maintain pain hypersensitivity after central sensitization has occurred (Latremoliere and Woolf, 2009). Therefore, an increased pain sensitivity in non-inflamed tissue can result and persist even after the peripheral stimulus is
removed and the tissues apparently healed (Latremoliere and Woolf, 2009). Synaptic inputs to CNS nociceptive neurones then are facilitated, potentiated or amplified as they are recruited and can produce an increase in the neuronal RF, threshold, spatial and temporal properties (Latremoliere and Woolf, 2009). This can also result in NS neurones converting into WDR neurones so that they now respond to both innocuous and noxious stimuli (Dubner and Ren, 2004; Latremoliere and Woolf, 2009; Sessle, 2000, 2011b; Woolf 2007; Woolf and King, 1990). Central sensitization can be initiated by intense noxious stimuli including repeated heat stimuli above 49 °C, electrical stimulation of C-fibres, chemical activation by irritants including MO, formalin or capsaicin, and nerve damage.

### 2.2.8.1 MO/Tooth pulp-evoked central sensitization

MO application to the rat tooth pulp has been shown to induce central sensitization (increased excitability) in nociceptive neurones of the MDH (e.g. Chiang et al., 1998, 2002, 2005, 2007, 2010; Iwata et al., 1998; Xie et al., 2007) and thalamus (Kaneko et al., 2005; Park et al., 2006; Zhang et al., 2006). This central sensitization is reflected as increased neuronal responses to innocuous and noxious stimuli, lowered activation thresholds, increased spontaneous activity and expanded RFs. Nociceptive inputs are also relayed to the trigeminal motoneurones within the cranial motor nuclei, including Vm, which supplies and regulates jaw muscles (Capra, 1995; Dostrovsky 2006; Paxinos, 2004; Sessle, 2000, 2006, 2009; van Steenberghe and de Laat, 1989). Although no study to date has documented directly the effect of MO application to the tooth pulp on the excitability of trigeminal motoneurones per se, the time frame of central sensitization induced by MO application to the tooth pulp is consistent with a concurrent increase in electromyographic activity (EMG) activity in the jaw muscles such as the anterior digastric (AD) and masseter muscles (Narita et al., 2012; Sunakawa et al., 1999; Tsai et al., 1999). Glial cells including astrocytes have been shown to be integrally involved in the MDH central sensitization (see below).
2.2.8.2 N-methyl-D-aspartate receptors, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)-propanoic acid receptors

A significant neurotransmitter crucial for central sensitization is glutamate, the major excitatory neurotransmitter in the CNS. Glutamate can act on numerous receptors in the spinal dorsal horn and MDH, including ionotropic amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (AMPARs), N-methyl-D-aspartate receptors (NMDARs), and kainate (KA) receptors as well as metabotropic (G-protein coupled) glutamate receptors (mGluR) (Dubner and Ren, 2004; Latremoliere and Woolf, 2009; Sessle, 2011b; Sessle, 2000). AMPARs and NMDARs are arranged in alternating fashion along almost every synapse in the superficial layers of the dorsal horn, while mGluRs are arranged along the postsynaptic density zone (PSD) (Antal et al., 2008; Latremoliere and Woolf, 2009; Pitcher et al., 2007).

NMDARs are composed of 4 subunits forming a tetramer, and consist of 2 low-affinity glycine-binding NR1 subunits and 2 of NR2A-D or NR3A/B subunits (Latremoliere and Woolf, 2009; Stephenson et al., 2008). Similarly, the AMPAR is a tetramer that commonly consists of GluR1 and GluR3, which are Ca^{2+} permeable, and GluR2, which is impermeable to Ca^{2+} (Latremoliere and Woolf, 2009; Polgar et al., 2008). The family of mGluRs is composed of 8 receptors that can be classified into 3 subgroups, determined by their coupling with specific G α -proteins (Conn et al., 1997; Latremoliere and Woolf, 2009). A magnesium (Mg^{2+}) ion blocks the NMDAR pore at resting conditions, but is released after membrane depolarization. Once the NMDAR pore is unblocked, glutamate binding to the receptor can allow influx of Ca^{2+} into postsynaptic neurones to activate intracellular pathways and gene transcription to maintain central sensitization (Latremoliere and Woolf, 2009; Mayer et al., 1984). Owing to the importance of NMDARs to central sensitization, the action of NMDAR antagonists including MK801 have been shown to prevent and reverse hyperexcitability of nociceptive neurones and central sensitization in the spinal dorsal horn and MDH (Chiang et al., 1998; Latremoliere and Woolf, 2009; Ma and Woolf, 1995; Woolf and Thompson, 1991). Another molecule critical for central sensitization is SP, which acts on the NK1 G-protein-coupled receptor. It is responsible for long-lasting membrane depolarization, and is potentiated by CGRP receptor (CGRPR) activation, leading to protein kinase A (PKA) and protein kinase C
PKC and PKA can then phosphorylate AMPAR subunit GluR1 residue Ser831 and Ser845, respectively; while they can phosphorylate Ser896 and Ser890/Ser897 on NMDAR (Latremoliere and Woolf, 2009; Leonard et al., 1997; Tingley et al., 1997). This phosphorylation can modify activity of these channels as well as lead to their trafficking to or from the membrane, and then influx of Ca^{2+} can activate intracellular pathways including the phospholipase C (PLC)-phosphatidylinositol-3-kinase (PI3 K) pathway, and the mitogen-activated protein kinase (MAPK) pathways involving extracellular signal-regulated kinases (ERK1 and ERK2) and cAMP response element binding protein (CREB) (Ji et al., 1999; Kawasaki et al., 2004; Okada-Ogawa et al., 2009; Pezet et al., 2008). Both ERK and CREB activation can induce translational and post-translational modifications that maintain central sensitization, including increasing NMDAR function through phosphorylation, trafficking of AMPAR onto post-synaptic membranes and increasing AMPAR and NMDAR currents to augment synaptic efficacy (Kohno et al., 2008; Latremoliere and Woolf, 2009; Slack et al., 2004).

2.3. Central control of orofacial motor function

2.3.1 Orofacial muscles

Orofacial motor functions can be separated into voluntary, involuntary, automatic, and semiautomatic functions that result from coordinated activity of orofacial muscles, including the face, jaw, tongue, palate, pharynx and larynx (Avivi-Arber et al., 2011).

Sensorimotor functions of the mouth and face, such as jaw opening, jaw closing, mastication, swallowing, speaking, require coordinated muscle activation. These muscles include muscles of the face, jaw, tongue, palate, pharynx and larynx. Muscles of jaw opening include the anterior digastric, geniohyoid, and mylohyoid while jaw-closing muscles are commonly the masseter, temporalis and medial pterygoid. These muscles are involved in mastication, and with suprahyoid muscles like the stylohyoid, are also important in swallowing, breathing and speaking (Avivi-Arber et al., 2011; Sessle, 2006; Van Eijden et al., 1997).
2.3.2 Elemental and semiautomatic orofacial motor functions

Voluntary orofacial sensorimotor functions include jaw opening, jaw closing and tongue protrusion, which can all be initiated consciously (Avivi-Arber et al., 2011). Brain regions involved in voluntary functions include face-M1, the pre-motor cortex, the supplementary motor area (SMA) of the cortex, the basal ganglia and the cerebellum (Avivi-Arber et al., 2011). Involuntary activities include the jaw-opening reflex, gag-reflex and cough, which are initiated subconsciously (Avivi-Arber et al., 2011) and utilize brainstem networks (see below). Semiautomatic functions include chewing and swallowing, and may be initiated consciously but maintained subconsciously by activation of regions of the brain such as the cortical masticatory area/swallow cortex, face-M1 and face-S1, and brainstem networks (Avivi-Arber et al., 2011).

2.3.3 Brainstem motor nuclei

Orofacial muscle activity can be initiated through brainstem networks, as well as descending innervation from face-M1 and face-S1 of brainstem cranial nerve motor nuclei. Brainstem networks include the central pattern generators, which are involved in involuntary movements, and modulation of semiautomatic muscle activities such as chewing and swallowing (Avivi-Arber et al., 2011; Sessle, 2006). The cranial nerve motor nuclei include the Vm supplying most jaw muscles, the nucleus ambiguus which innervates palatal, laryngeal and pharyngeal muscles through the vagus and glossopharyngeal nerves, and the hypoglossal nucleus which innervates the extrinsic and intrinsic muscles of the tongue (Avivi-Arber et al., 2011). Face-M1 and face-S1 may have excitatory or inhibitory actions on brainstem motor nuclei, while interneurones between face-M1 and face-S1 modulate each area’s activity and play a role in cortical neuroplasticity (Avivi-Arber et al., 2011) as outlined below. In the case of jaw motor control, there are interneurones also in the supratrigeminal nucleus and the intertrigeminal area that synapse onto Vm, and these premotor neurones receive innervation from both sensory afferents and M1 output neurones (van Steenberghe and De Laat, 1989). As well, interneurones from the contralateral Vc and bilaterally from the Vo have been found to terminate in Vm, suggesting that jaw muscle activation can be
modulated by sensory information at the level of the medulla (van Steenberghe and De Laat, 1989).

### 2.3.4 Primary motor cortex

M1 is crucial for the initiation of volitional movements, and its activity is coordinated with subcortical structures like the brainstem and diencephalon which provide feedback control signals to modulate movement (Avivi-Arber et al., 2011; Canedo, 1996). With the use of stimulation techniques, including intracortical microstimulation (ICMS, see below), it was discovered that M1 is organized into a contiguous map containing a representation of the body (for review, see Dum and Strick, 2005). Anatomical studies have demonstrated connections from premotor areas to M1, including the SMA and the cingulate motor area (CMA) (Dum and Strick, 2005). While stimulation studies have shown that these areas can generate body movements, stimulation in M1 has the lowest threshold for evoking movement and the highest probability for evoking movement than any other cortical area (Dum and Strick 2002, 2005).

In macaque monkeys, ICMS studies have revealed that distal body areas, such as the digits and wrist, are represented at more than one location in M1, while proximal areas such as the elbow surround the M1 loci representing the digits; this arrangement is thought to allow a muscle to be activated in multiple synergies with different joints (see Dum and Strick, 2005; Scheiber, 2001). M1 contains 6 horizontal layers (I-VI), with layer V containing large pyramidal cells that project to other brain areas, but most importantly to the brainstem motoneurones involved in orofacial movement. The corticofugal fibres of the pyramidal tract are one of the main outputs of the M1 (Avivi-Arber et al., 2011; Canedo, 1996; Scheiber 2001). The pyramidal tract is required for skilled locomotion, as lesioning of the cat’s bulbar pyramids prevents any skilled task such as ladder walking (Liddel and Phillips, 1944), and damage to face-M1 or tracts involved in orofacial movement can lead to loss of skilled orofacial motor control or impaired mastication and swallowing, or other dysfunctions such as in amyotrophic lateral sclerosis in humans (Avivi-Arber et al., 2011; Sessle et al., 2007; Urban et al., 1998). Stimulation of the face-M1 by electrical or magnetic transcranial stimulation produces motor evoked potentials (MEPs) and movements of jaw closing, jaw
opening and tongue protrusion (Avivi-Arber et al., 2011; Murray and Sessle, 1992; Huang et al., 1988). 30% of direct, fast-conducting corticobulbar fibres innervate contralateral motoneurones, but the suprahyoid musculature, masseter and digastric activation is bilateral (Nordstrom et al., 1999; van Steenberghe and De Laat, 1989).

2.3.5 Orofacial muscle representations in face-M1 and face-S1

The ICMS technique has been used to map the cortical representation of the orofacial muscles in the rat and monkey, and the role of face-M1 in orofacial motor function across species has been documented (Avivi-Arber et al., 2010a, 2010b; Sessle et al., 2007). Studies mapping the representations of orofacial muscles in monkeys with ICMS (0.2 msec pulses, 333 Hz, 35 msec) have revealed a general organization of representations of the contralateral jaw and tongue musculature surrounded rostrally, medially and caudally by representations of the facial musculature (Huang et al., 1988, 1989a). This stimulation train evokes elemental movements such as jaw-opening, while longer stimulations (0.2 msec pulses, 50 Hz, 3s) can evoke masticatory-like and swallowing movements (Huang et al., 1989b; Narita et al., 1999, 2002; Sessle, 2005). Extracellular recordings in monkeys support the finding that face-M1 neurones are involved in chewing and swallowing (Arce-McShane et al., 2014; Martin et al., 1997; Yao et al., 2002). Studies using functional magnetic resonance imaging (fMRI) in humans have demonstrated M1 activity in swallowing and tongue movements (Martin et al., 2001, 2004). Multiple representations of a particular orofacial muscle were found in face-M1, suggesting a similar functional and synergistic organization as in limb-M1 (Huang et al., 1988; Dum and Strick, 2005). Further evidence for the role of face-M1 in evoking orofacial muscle activation is comes from studies of bilateral cold block of face-M1, which prevents successful performance of novel tongue-protrusion tasks, and also alters mastication (Sessle, 2007; Yamamura et al., 2002). Similarly in face-M1 of rats, there is a general somatotopic representation of the orofacial muscles, with large representations of the anterior digastric and genioglossus muscles in face-M1 of both hemispheres of the cortex, although a large contralateral predominance exists for the left and right anterior digastric muscles (Avivi-Arber et al., 2010a; Donoghue and Wise, 1982).
Face-S1 has a somatotopic representation of orofacial somatosensory inputs from cutaneous and deep tissue, but may also play a role in motor control (Avivi-Arber et al., 2011; Sessle, 2005). ICMS can evoke orofacial muscle activity also in the face-S1 of rats and primates, as well as being involved in mastication and swallowing (Avivi-Arber et al., 2010a; Donoghue and Wise, 1982; Huang et al., 1989a; Narita et al., 1999, 2002). Extracellular recordings have corroborated the findings from ICMS studies, where neurones in face-S1 can also be activated during tongue movement, chewing and swallowing (Huang et al., 1989a; Lin et al., 1994a, 1994b, 1994c; Yamamoto et al., 1988). This control of motor activity by face-S1 is also suggested through the intracortical connections between S1 and M1, and because face-M1 also receives inputs from deep and superficial tissues, both face-S1 and face-M1 may be important in controlling orofacial muscle activity (Avivi-Arber et al., 2011; Sessle, 2005, 2006).

2.3.6 Sensory inputs to motor regions controlling orofacial function

Sensory inputs from orofacial receptors including mechanoreceptors, proprioceptors, thermoreceptors, nociceptors and chemoreceptors can modulate the output of brainstem motorneurons (Avivi-Arber et al., 2011). Sensory input reaching the VBSNC and solitary tract nucleus in the caudal brainstem can relay directly or indirectly through the intertrigeminal nucleus, supratrigeminal nucleus and reticular formation to adjacent brainstem motorneurons (Avivi-Arber et al., 2011). As well, VBSNC and solitary tract nuclei may project through the thalamus to face-S1 and face-M1, which have descending projections to brainstem motorneurones to modulate orofacial motor activity (Avivi-Arber et al., 2011).

M1 receives somatosensory information in layers III and V from direct projections from the thalamic motor nuclei, but also from intracortical connections from layer IV of S1 (for review, see Asanuma, 1989; Avivi-Arber et al., 2011; Monkeys: Rausell and Jones, 1995; Rats: Aldes, 1988; Cicirata et al., 1986a, 1986b; Donoghue et al., 1979; Miyashita et al., 1994). In the rat, most intracortical connections to face-M1 are from the vibrissa-S1 (Izraeli and Porter, 1995; Porter, 1996), although in primates, studies have demonstrated that face-
M1 receives projections from area 3a, which contains direct proprioceptive inputs from muscle spindles and indirect exteroceptive inputs from areas 3b and 1 (Iyengar et al., 2007; for reviews, see Kaas et al., 2006).

### 2.3.7 Neuroplasticity following orofacial manipulations

Plasticity in the brain was first discovered in the hippocampus when long term potentiation of synapses by coupling of presynaptic and postsynaptic responses increased excitatory postsynaptic potentials (EPSPs) and induced synaptic plasticity (Bliss and Lomo, 1973). A larger scale plasticity is reflected in the cortex by reorganization of cortical sensory representations, or cortical maps (Sanes and Donoghue, 2000). This cortical reorganization may occur after afferent denervation, causing the neurones that have lost innervation to respond to neighbouring inputs. Cortical reorganization can also be experience-dependent, where training of limb motor function may lead to an increase in the cortical representation of the limb (Sanes and Donoghue, 2000). Orofacial manipulations, such as whisker trimming, extractions, implants and motor learning have been shown to alter cortical representations in the face-S1 and face-M1 (see Avivi-Arber et al., 2011). For example, although unilateral trimming of the whiskers may not alter representations within face-M1, bilateral trimming for 5 days can modify vibrissal and limb representations in the face-M1 (Huntley, 1997; Keller et al., 1996). Extraction of the incisor in rats has also been shown to modulate cortical representations, with increases in anterior digastric representation in the face-M1 as well as representations of the tongue, chin, gums and buccal pad in face-S1 (Avivi-Arber 2009; Avivi-Arber et al., 2010b; Henry et al., 2005). Face-M1 activation is also increased when edentulous patients are treated with implant-supported complete dentures instead of traditional complete dentures (Yan et al., 2008). In humans, learning and acquisition of motor skills can alter face-M1 excitability, as learning a tongue-protrusion task increases face-M1 excitability as measured by TMS (Boudreau et al., 2007, 2010). Local anaesthesia applied periorally or intraorally can induce cortical reorganization of orofacial RFs within face-S1 (Katz et al., 1999; Nicolelis et al., 1993).
2.3.8 Neuroplasticity of face-M1 associated with pain conditions

Numerous studies have demonstrated a change in excitability of M1 after noxious stimulation of the limb and orofacial tissues. Acute pain studies in humans have demonstrated that pain evoked by laser heat, capsaicin or hypertonic saline can all attenuate motor responses elicited by TMS (Farina et al., 2001; Le Pera et al., 2001; Mercier and Leonard, 2011). Similarly in the orofacial region, experimentally induced pain has been shown to decrease face-M1 excitability, manifested as increased thresholds for evoking movement by TMS in humans or ICMS in animals (Adachi et al., 2008; Boudreau et al., 2007). Imaging studies in humans have also shown structural changes accompanying orofacial pain, where trigeminal, neuropathic and idiopathic facial pain is associated with decreases in grey matter in the face-M1 (DaSilva et al., 2008; Nash et al., 2010b; Schmidt-Wilcke et al., 2010). However, no study has examined face-M1 neuroplasticity in association with dental pain.

3. The intracortical microstimulation technique

ICMS and EMG recordings of cortically evoked muscle activity are techniques that have been used to study the neuroplastic capacity of the sensorimotor cortex (Adachi et al., 2008; Asanuma 1989; Neafsey et al., 1986). Alterations to the orofacial environment (some which can lead to pain) can induce cortical plasticity and alter orofacial behavior, and ICMS has been used to measure this cortical plasticity (Adachi et al., 2008; Avivi-Arber et al., 2011). Techniques such as fMRI, positron emission tomography (PET) and TMS have been used to map out the organization of the M1 (Sanes and Donoghue, 2000).

In ICMS experiments, a stimulating electrode is inserted into the depth of the cortex to electrically stimulate pyramidal tract neurones projecting to the brainstem and spinal cord (Asanuma, 1989). This allows for greater spatial resolution compared to experiments where only the surface of the cortex is stimulated. At ICMS threshold intensity, stimulation can excite the most excitable neurones (myelinated, larger diameter) to evoke a short-latency muscle response. The ICMS threshold intensity is the lowest stimulus intensity that evokes
ICMS-evoked muscle activity by activating the most excitable projection neurones at the tip of the stimulating microelectrode.

3.1 Effective extent of ICMS current spread

The spatial extent of neural activation is determined by two main factors: the effective spread of current, which is proportional to the square root of the current intensity, and the excitability of the neural elements, with the nodes of Ranvier and initial segments of axons being the most excitable due to their high concentration of sodium channels (Clark et al., 2011; Tehovnik et al., 2006). The effective spread of current refers to the distance at which a current of a given intensity directly activates neuronal elements (Nowak and Bullier, 1996). By stimulating M1 with 4.0 µA single pulse ICMS currents of 0.2 ms durations, monosynaptic excitatory postsynaptic potentials can be recorded in neurones within 0.5 mm of the microelectrode tip (Asanuma and Rosen, 1973).

ICMS can also excite pyramidal tract neurones trans-synaptically, which is evident with repetitive ICMS at frequencies between 200 and 400 Hz that lead to temporal summation, such as used in this thesis (Asanuma et al., 1976; Jankowska et al., 1975; Schieber et al., 2001). Pyramidal neurone activation by repetitive ICMS can then initiate muscle responses that can be measured by EMG recordings. While a 20 µA ICMS current (0.2 ms pulse, 300 Hz) can activate pyramidal tract neurones within a radius of 0.25 mm (Sapienza et al., 1981), a 50 µA ICMS current (0.2 ms pulse, 300 Hz) can activate pyramidal tract neurones within a 0.5 mm radius (Neafsey et al., 1986; Ranck, 1975; Tehovnik et al., 1996, 2006). Furthermore, axon collaterals can produce excitatory postsynaptic potentials in M1 pyramidal tract neurones within a 1-2 mm radius (Asanuma and Rosen, 1973). However, since many interneurones are inhibitory, they can also be activated and limit the effective spread of ICMS current (Asanuma et al., 1976).

3.2 Effect of general anaesthesia

In acute ICMS experiments involving the use of a general anaesthetic, the ICMS threshold intensity of ICMS evoked muscle activity can be modulated by anaesthetic level and muscle position or stretching (Asanuma et al., 1968; Graziano et al., 2002b; Sessle and
Wiesendanger, 1982; Wong et al., 1978). Ketamine is often used as a general anaesthetic in such experiments as it does not inhibit ICMS-evoked muscle responses (Nudo et al., 2003). Ketamine is a non-competitive antagonist NMDARs, which are found primarily on dendrites and cell bodies of neurones (Morgan and Curran, 2012; Yamakura et al., 2001). Although deep states of anaesthesia may increase thresholds for ICMS-evoked muscle activation (Gioanni and Lamarche, 1985; Sapienza et al., 1981; Tandon et al., 2008), low-intensity ICMS produces similar EMG responses and M1 maps in both anaesthetized and awake animals (Huang et al., 1989b; Sapienza et al., 1981; Tandon et al., 2008). Since ICMS primarily stimulates axons, ketamine has a small effect on ICMS parameters and is generally considered an appropriate anaesthetic for ICMS studies as long as a stable level of general anaesthesia is achieved (Nowak and Bullier, 1998a, 1998b).

4. Glial cells

In the peripheral nervous system (PNS) and CNS, non-neuronal cells have numerous functions including nurturing neurones, replenishing chemical imbalances and repairing neurones after injury or inflammation. Subtypes of glial cells in the CNS include astrocytes, microglia and oligodendrocytes, while the PNS contains Schwann cells and satellite glial cells (Chiang et al., 2011; Haydon and Carmignoto, 2006; Volterra and Meldolesi, 2005).

4.1. Microglia

Microglia are glial cells that originate from the bone marrow, and migrate through the circulatory system into the CNS to function in immune responses (Kettenmann et al., 2010). Visualizing microglia can be accomplished with staining by targeting cell-surface associated molecules or intracellular molecules such as isolectin B4 (ILB4), tomato lectin or ionized calcium-binding adapter molecule 1 (Iba1) (Kettenmann et al., 2010). Although they can freely migrate into the CNS until postnatal development, they cannot enter the brain afterwards until blood-brain barrier damage (Milder et al., 2007). In the brain, spinal cord, eye and optic nerve, the population of microglia reside in a “resting” state, where they have a ramified morphology consisting of a small soma and fine cellular processes (Kettenmann et al., 2010). After blood-brain barrier damage however, monocytes from the bone marrow can
infiltrate the brain to transform into microglia (Milder et al., 2007). The microglia then become activated, thickening and retracting their processes to become more amoeboid-like, and then can be stained with Iba-1 antibodies, (Block et al., 2007; Colton and Wilcock, 2010; Kettenmann et al., 2010; Zhuo et al., 2011). Activated microglia can then function in the immune response, proliferating and recruiting additional microglia to the injury site while also releasing multiple proinflammatory and immunoregulatory factors and compounds (Kettenmann et al., 2010). Microglia can also become activated due to infection, ischaemia, neurodegenerative diseases or any alteration in the homeostasis of the CNS, including pain (Kettenmann et al., 2010).

In the spinal cord and MDH, microglia play a significant role in modulating peripheral inflammation and neuropathic pain (Mostaeezur et al., 2012; Trang and Salter, 2012; Xie et al., 2007; Zhuo et al., 2012). When activated, microglia upregulate surface markers and membrane-bound proteins including CD11b, P2X4 receptors, toll-like receptor 4, CD44 and MHC II (Zhuo et al., 2012). ATP release after tissue injury can activate microglia through their P2 purinergic receptors, which induce microglial release of cytokines, chemokines and other neurotrophic factors to modulate neuronal function (Trang and Salter, 2012). P2X4 receptors, which are activated by ATP, are necessary for tactile allodynia after nerve injury, and can be upregulated by the chemokine CCL21, which is released by injured neurones, the cytokine interferon γ, tryptase from activated mast cells, and extracellular matrix molecule fibronectin (Tsuda et al., 2004; Trang and Salter, 2012). P2X4 activation on microglia can then activate intracellular p38-MAPK pathways and induce microglial release of brain derived neurotrophic factor (BDNF), which acts on tyrosine receptor kinase type b (trkB) receptors on spinal laminae 1 neurones to induce a depolarizing shift in neuronal membrane potential, so that GABA evoked responses become depolarizing, instead of hyperpolarizing. This neuronal mechanism can then account for the phenotypic switch of spinal laminae 1 neurones in mechanical allodynia, when innocuous mechanical input can then produce spinal laminae 1 neuronal depolarization and signal pain in higher brain areas (Coull et al., 2005).

p38-MAPK activation has also been shown in inflammatory pain following intradermal injection of formalin, where p38-MAPK expression was only colocalized with microglia in the superficial dorsal horn (Svennsson et al., 2003). Phosphorylation of p38-MAPK induced by formalin injection subcutaneously in the hind foot of rats could be blocked by spinal
injection of anti-inflammatory cytokine IL-10, which reduced nociceptive behavior (Zhou et al., 2007). Inhibition of p38-MAPK by SB203580 or minocycline can attenuate nociceptive responses induced by MO application to the tooth pulp or carrageenan-induced hyperalgesia in the paw, respectively (Hua et al., 2005; Xie et al., 2007). Inflammatory pain may activate microglia in a different pathway as well, as intracellular caspase-6 (CASP6), a neuronal regulator of apoptosis and axonal degeneration, may activate microglia after inflammatory pain by intraplantar injection of formalin or bradykinin (Berta et al., 2014). This CASP6-dependent mechanism may act through TNF-α, release of which increases excitatory postsynaptic currents (EPSCs) in spinal cord neurones (Berta et al., 2014). Taken together, these results suggest that microglia play a key role in inflammatory pain conditions, which has been shown as minocycline, a microglial inhibitor, application to the spinal cord blocks carrageenan-induced hyperalgesia (Hua et al., 2005). Microglia activation after nerve injury in the rat has been shown in supraspinal regions as well, including the VPL of the thalamus, basal ganglia, insula, S1 and ACC (Di Cesare Mannelli et al., 2013).

4.2 Oligodendrocytes

Oligodendrocytes in the CNS, and Schwann cells in the PNS, function to ensheath axons in myelin to increase axonal conduction velocity (Sherman and Brophy, 2005). Oligodendrocytes differ in their function compared to Schwann cells as oligodendrocytes can myelinate multiple axons simultaneously, while Schwann cells are limited to a single axon (Nave, 2010; Sherman and Brophy, 2005). Myelin acts as an insulator, allowing the action potential to propagate along neurones with very little loss of current. Along the myelinated axon are sites where there is no myelin, termed nodes of Ranvier, where a high concentration of sodium channels are present to allow for membrane depolarization to continue conduction of the nerve impulse (Nave, 2010; Sherman and Brophy, 2005). This form of propagation is termed saltatory conduction. Demyelination can occur in disease states of the CNS, such as multiple sclerosis, or in the PNS as charcot-marie-tooth disease (Nave 2010, Noseworth, 1999; Sherman and Brophy, 2005)
4.3 Astrocytes

Astrocytes are non-neuronal glial cells in the brain that were initially believed to be involved in structurally supporting neurones as a scaffold. However, research has provided evidence for their role in synapse modulation, synapse formation, adult neurogenesis, brain vascular tone, guiding development, regulating the extracellular concentrations of ions, metabolites and neurotransmitters (Keyser et al., 1995, Volterra and Meldolesi, 2005; Walz, 1989). These glial cells can be labeled by intermediate filament protein, glial fibrillary acidic protein (GFAP), S100 calcium-binding protein β (S100β), which has provided imaging data demonstrating that astrocytes are orderly arranged to contact a specific area of microvasculature and thousands of synapses (Bushong et al., 2002; Hewet, 2009). Astrocytes, like neurones, release chemical mediators and can be activated and send signals to neighbouring cells, which is referred to as gliotransmission, but unlike neurones, they cannot generate action potentials. Their activation can be spontaneous, but also can depend on chemical signals from neuronal circuits, termed neurone-dependent excitation (Volterra and Meldolesi, 2005). Neurone-dependent excitation can result from neuronal release of various transmitters and factors such as glutamate, GABA, acetylcholine, noradrenaline, dopamine, ATP, nitric oxide and BDNF. Gliotransmission through astrocyte membranes can occur through hemichannels, which are hexamers of connexin 43 (CX43) and connexin 30 (CX30) that form gap-junctions when coupled with adjacent hemichannels of neighbouring astrocytes (Chiang et al., 2011, 2012; Volterra and Meldolesi, 2005).

Astrocyte activation, whether in CNS ischaemia, trauma or neurodegeneration, induces hypertrophy of cellular processes, and upregulation of GFAP and vimentin, and reexpression of nestin, which are intermediate filaments found in astrocytes. These intermediate filaments provide the structural support for astrocytes to form syncytium networks after activation and perform protective functions, such as glutamate uptake from the extracellular space and K+ buffering (Pekny and Nilsson, 2005). Excessive glutamate elevations in the extracellular space can cause excitotoxicity that is linked to neurological diseases including amyotrophic lateral sclerosis and epilepsy (Anderson and Swanson, 2000).
Astrocyte activation can induce intracellular signaling cascades involving the ERK and c-Jun N-terminal kinase (JNK), leading to synthesis of inflammatory factors IL-1β, IL-6, TNF-α, nitric oxide (NO). IL-1β, IL-6, TNF-α can act on toll-like receptors (TLRs) on other astrocytes to induce activation, and produce a feed-forward activation of astrocytes (Milligan and Watkins, 2009). IL-1β, IL-6, TNF-α can also downregulate glutamate uptake and increase conductivity of AMPAR and NMDARs in neurones, both ultimately increasing second-order neurone excitability in the spinal cord (Milligan and Watkins, 2009). These proinflammatory factors may either increase excitatory synaptic transmission or decrease inhibitory synaptic transmission, as well as induce long term synaptic plasticity (Kawasaki et al., 2008).

Neuronal inputs can modulate intracellular Ca2+ ([Ca2+]i) oscillations in astrocytes, which vary in amplitude, frequency and propagation. Spontaneous [Ca2+]i oscillations are more frequent in development, but can occur in adulthood when inositol triphosphate (IP3) receptors in astrocytes are activated, as well as through influx by voltage gated channels (Volterra and Meldolesi, 2005). Intercellular [Ca2+]i oscillations have a varying range, and can involve a small population of cells but increase to hundreds of micrometres (Volterra and Meldolesi, 2005).

Although neurone-astrocyte signaling can occur at synapses, commonly named a tripartite synapse, astrocytes have also been found to modulate activity along axons and white matter in which case they are referred to as fibrous astrocytes. It has been shown that axons release neurotransmitters such as glutamate onto glial cells such as oligodendrocyte precursors (Kukley et al., 2007; Ziskin et al., 2007). As well, fibrous astrocytes have been demonstrated to propagate Ca2+ signals in response to neurotransmitters and axonal activity (Butt et al., 2004). Further studies have demonstrated a coupling between axons and fibrous astrocytes, where ATP release after axonal electrical activity mediates Ca2+ excitability in astrocytes to release ATP and glutamate, and propagate Ca2+ waves (Hamilton et al., 2008).

A process specific to astrocytes is the glutamate/glutamine shuttle that replenishes glutamate in excitatory neurones and the GABA/glutamate/glutamine shuttle that replenishes GABA in inhibitory neurones. The shuttle removes excessive glutamate (or GABA) from the synaptic
cleft, thereby collecting glutamate to be converted into glutamine by GS in the astrocyte. Glutamine is then transported back to the presynaptic terminal where it is converted into glutamate in excitatory synapses and into GABA in inhibitory synapses to replenish the supply of neurotransmitters (for review, see Chiang et al., 2012; Danbolt, 2001; Hertz and Zielke, 2004; Kanamori and Ross, 2006; Verkhratsky and Butt, 2013). Inhibition of the shuttle at inhibitory synapses will impede inhibitory transmission; and inhibition of the shuttle at excitatory synapses will impede excitatory transmission (Hertz and Zielke, 2004; Jiang et al., 2012; Miyake and Kitamura, 1992; Norenberg and Martinez-Hernandez, 1979; Verkhratsky and Butt, 2013). GS is exclusively expressed in astrocytes and MSO is a potent and specific inhibitor of GS (Miyake and Kitamura, 1992; Norenberg and Martinez-Hernandez, 1979; Verkhratsky and Butt, 2013).

Astrocytes are found in all regions of the CNS, and in the hippocampus and cerebral cortex, one astrocyte may contact more than 100,000 synapses (Sofroniew and Vinters, 2010). There may be regional differences in astrocyte protein expression profiles across the CNS (Hewett 2009). For example, the epidermal growth factor receptor (EGFR) in forebrain astrocytes may regulate neurodegeneration, though deletion of the gene has no effect on midbrain astrocytes (Wagner et al., 2006). Similarly, expression of Cx43 and Cx-40 in S1 of mice was correlated with barrel cortex, where septal regions had decreased expression of both connexins (Houades et al., 2008).

**4.4. Glial cell involvement in pain conditions**

After peripheral nerve injury or inflammation of peripheral tissues, hyperalgesia has been shown to be accompanied with activation of satellite glial cells, astrocytes and microglia in the PNS and CNS (Chiang et al., 2011, 2012; Xie et al., 2007; Zhang et al., 2007, 2009). In the spinal dorsal horn and MDH, the timing of activation of astrocytes and microglia can vary, although astrocytes are often activated first in acute inflammatory pain, while activation of microglia follows (Chiang et al., 2011, 2012; Qin et al., 2006). Neuropathic pain models of paw incision, chronic constriction injury, L5 nerve ligation and transection showed increases in spinal cord astrocyte GFAP expression as well as spinal cord microglia Iba-1 expression (Colburn et al., 1999; Garrison et al., 1991; Romero-Sandoval et al., 2008).
Inflammatory pain models showed similar activation of astrocytes and microglia in the spinal cord (Chiang et al., 2011; Qin et al., 2006). In the spinal cord, inhibition of aconitase by fluoroacetate (FA) can attenuate thermal and mechanical hyperalgesia and mechanical allodynia after zymosan-injection (Meller et al., 1994). Propentofylline, which inhibits adenosine transport cyclic-adenosine-5’,3’-monophosphate (cAMP)-specific phosphodiesterase (PDE IV) in astrocytes, can decrease mechanical allodynia and hyperalgesia after L5 nerve transection if applied systemically, with spinal cord sections displaying decreased GFAP and microglial activation (Sweitzer et al., 2001; Tawfik et al., 2007).

The role of astrocytes in orofacial pain has been implicated by numerous studies where astrocyte inhibition in the MDH reduces mechanical and thermal hypersensitivity and hyperalgesia after orofacial nerve injury or inflammation (Abbadie et al., 2009; Chiang et al., 2001, 2012; Milligan and Watkins, 2009; Ren and Dubner, 2010; Sessle, 2011b). Models of orofacial inflammatory pain and neuropathic pain, including MO application to the tooth pulp, inferior alveolar transection, and infraorbital nerve injury, have been shown to induce central sensitization in CNS nociceptive neurones of the MDH (Chiang et al., 1998, 2002, 2005, 2007, 2010, Okada-Ogawa et al., 2009; Xie et al., 2007) and thalamus (Kaneko et al., 2005; Park et al., 2006; Zhang et al., 2006). This central sensitization can be modulated by astrocytes as increases in GFAP staining in the medulla has been demonstrated in orofacial inflammatory pain models, and medullary astrocyte inhibition by methionine sulfoximine (MSO) or FA can prevent or reverse the increased neuronal responses to innocuous and noxious stimuli, lowered activation thresholds, increased spontaneous activity and expanded RFs (Chiang et al., 2007; Okada-Ogawa et al., 2009; Mostafeezur et al., 2014; Tsuboi et al., 2011; Varathan et al., 2014; Xie et al., 2007). MSO inhibits the astrocytic enzyme glutamine synthetase (GS) (which functions in the glutamine-glutamate shuttle), and its application in the MDH can prevent or reverse inflammatory tooth pulp-induced mechanical hypersensitivity in the orofacial region to near baseline levels (Chiang et al., 2007; Tsuboi et al., 2011). FA inhibits aconitase, which functions as an enzyme in the astrocyte Krebs cycle, and its application in the MDH also produces similar reversals after inflammatory tooth pulp stimulation and trigeminal nerve injury (Cao et al., 2013; Okada-Ogawa et al., 2009; Xie et al., 2007). Similarly, microglial inhibition in the MDH and upper cervical spinal cord has
been demonstrated to reduce mechanical allodynia and hyperalgesia associated with orofacial inflammatory and neuropathic pain (Kiyomoto et al., 2013; Shibuta et al., 2012; Shimizu et al., 2009).

Astrocyte and microglial activation after neuropathic and inflammatory pain has also been shown in supraspinal areas including the ventrolateral PAG, amygdala, thalamus, and cortical areas such as the ACC and S1 (Chen et al., 2012; Di Cesare Mannelli et al., 2013; Ikeda et al., 2013; Varathan et al., 2014). In M1, glial cell involvement has been less reported, as proton magnetic resonance spectroscopy (MRS) has only found a correlation between chronic back pain and glial cell activation in the M1 as measured by the MRS glial marker myo-inositol, though acute inflammatory dental pain may induce glial activation in M1 (Sharma et al., 2012; Varathan et al., 2014).

5. Statement of the problem, hypothesis and study objectives

Dental inflammatory pain is one of the most common reasons patients seek dental or medical treatment, and is often associated with disruption of orofacial musculoskeletal functions. Application of the inflammatory irritant MO to the dental pulp in rats has been used to study central mechanisms associated with pulpal inflammation and pain. These studies have shown that pulpal MO application produces an increased neuronal excitability (central sensitization) in MDH reflected in increased neuronal mechanoreceptive field size, orofacial-evoked responses and activation thresholds. The MDH projects to higher brain centres and provides somatosensory afferent input that reaches face-M1. Immunolabeling studies have shown that tooth pulp inflammation in animals leads to increased astroglial activation in the MDH and that the associated MDH central sensitization can be normalised by medullary administration of MSO, a specific astrocytic inhibitor of the glutamate/glutamine shuttle that converts glutamate to glutamine. Astrocytes are non-neuronal cells that maintain homeostasis in neurones, but also respond in pain states by releasing inflammatory cytokines and neurotransmitters. Experimentally induced orofacial pain is associated in animals and humans with decreased face-M1 excitability manifested as increased ICMS or TMS thresholds for evoking EMG activity in orofacial muscles. However, it is unclear whether
MO application to the rat tooth pulp is also associated with altered face-M1 excitability and whether these changes involve VBSNC astrocyte function.

5.1. Hypotheses

The hypotheses were:

1. Noxious stimulation by MO application to the tooth pulp will result in decreased face-M1 excitability reflected as an increased ICMS threshold intensity required to evoke EMG activity in the right anterior digastric muscle (RAD).

2. Subsequent application of astrocyte inhibitor MSO to the MDH will attenuate the MO-induced changes in face-M1 excitability.

5.2. Objectives

Our specific aims were to use ICMS to test whether:

1. Application of the inflammatory irritant MO to the rat tooth pulp affects face-M1 excitability manifested as an altered ICMS threshold required to evoke EMG activity in the RAD.

2. Subsequent application of the astrocyte inhibitor MSO to the MDH can influence the MO-induced effects in face-M1.
Chapter 2

Materials and Methods

The University of Toronto Animal Care Committee approved all experimental procedures, in agreement with the regulations of the Ontario Animals for Research Act (R.S.O. 1990) and the Canadian Council on Animal Care Guidelines.

1. Animals

This study was conducted on young-adult male Sprague-Dawley rats (300-400 g). Adult male rats were used to maintain a uniform age and sex among animals, as both variables have been shown to affect oral motor behavior, while estrogen cycling can modulate nociceptive responses (Boudreau et al., 2007; Cairns et al., 2001a, 2007; Franchi et al., 2006; Greco et al., 2012; Hattemer et al., 2007; Huntley, 1997; Jonasson, 2005; Keller et al., 1996; Peyron et al., 2004; Sanders and Slade, 2011; Shiga et al., 2012; Yao et al., 2002; Youssef et al., 1997; Zhang et al., 2012).

Consistent with standard experimental procedures in the laboratory (Adachi et al., 2007, 2008; Avivi-Arber et al., 2010a, 2010b), rats were singly housed in individual cages (27 x 45 x 20 cm) to ensure adequate food and water intake and fed a soft diet (Rodent Diet No. 2018M; Harlan Teklad). The cages contained a polyvinyl chloride tube (used as a shelter and gnawing device), with temperature and humidity maintained at optimal levels (21 ± 1 °C and 50 ± 5 %, respectively). Rats were exposed to a 12-hour light/dark cycle (lights on at 07:00 am). Diet consistency may modify somatosensory inputs to face-S1 and face-M1, thereby affecting jaw and tongue muscle activities (e.g. Inoue et al., 2004; Lund and Kolta, 2006; Okayasu et al., 2003). However, it has no significant effect on face-M1 motor representations (Avivi-Arber et al., 2010a, 2010b).

2. Study Groups and design

After all surgeries, rats were monitored for 30 mins to determine a stable state of anaesthesia and stable EMG activity in the RAD (jaw opening) muscle (Fig. 2-1). Then, ICMS
(described in section 4.0) threshold intensity for evoking RAD EMG activity was measured after 15 mins and 30 mins to determine and confirm stable baseline RAD thresholds. Then, MO (‘MO group’, n=16) or vehicle control [mineral oil, ‘MIN group’ (n=8) or phosphate-buffered saline, ‘PBS group’ (n=8)] was applied to the molar tooth pulp, and ICMS was delivered at 5 min intervals for 15 mins. Since there were no statistically significant differences in RAD thresholds between the MIN and PBS groups (see Results) their data was pooled into a ‘vehicle group’. Preliminary studies revealed that MO-induced changes in RAD thresholds peaked at 15 mins following MO application to the molar tooth pulp. Therefore, 15 mins following MO or vehicle application to the molar tooth pulp, MSO [‘MO/MSO group’ (n=8), ‘vehicle/MSO group (n=8)] or PBS control [‘MO/PBS group’ (n=8), ‘vehicle/PBS group’ (n=8)] was applied topically to the caudal medulla. Thereafter, ICMS was delivered every 10 mins for the subsequent 120 min observation period. A ‘naïve’ group (n=8) received no dental treatment, no cervical laminectomy and no MO, MIN or PBS application to the molar tooth pulp or PBS or MSO to the medulla. Each experiment (Fig. 2-1) in experimental and control animals was alternatingly performed to reduce potential experimenter bias. Two investigators (HP and LA) carried out all experimental procedures and blinded data analyses, and followed strict standard protocols to ensure consistency in procedures and analyses.
3. Rat preparation:

3.1 Anaesthesia

General anaesthesia was induced in rats by intramuscular (i.m.) injection of 175 mg/kg of ketamine HCl (Ketaset®, Ayerst Veterinary Laboratories, Ontario, Canada) and 25 mg/kg of xylazine into the hindlimb. Anaesthesia was maintained with a femoral intravenous (i.v.) infusion regulated by a pump (PHD 2000, model 11 Plus, Harvard Apparatus, Inc., Holliston, MA, USA) at a rate of 75 mg/kg/hr of ketamine during the EMG electrode implantation and craniotomy, and 25 mg/kg/hr during the ICMS experiment (that could last up to 4 hrs) so that the rat was maintained in an anaesthetic depth that retained face-M1 excitability. At this anaesthetic depth, jaw and tongue muscles had at least 4-5 spontaneous twitches per minute and a noxious pinch applied to the hindpaw did not evoke a withdrawal reflex. Areas requiring incision were shaved with a fur trimmer and injected with lidocaine [0.1 ml, 2% Lidocaine in 1:100,000 epinephrine (Lignocaine, Lignospan standard®, Septodont, Ontario, Canada)] to block sensory inputs from the surgical sites to the brainstem, S1, M1 and other regions that through DNIC, could potentially modulate nociceptive inputs from the pulp or recruit descending influences from higher brain centres, thereby affecting cortical and subcortical excitability (Sessle, 2000; van Wijk and Veldhuijzen, 2010). The body temperature of the rat was maintained at 37.5 °C with a rectal thermometer controlled heating pad (Model 73A, YSI, Ohio, USA). Heart rate (>333-430 beats/min) and blood O₂ (>90%) levels were monitored with a pulse oximeter (8600, Nonin, MN, USA) consistent with previous experiments (Adachi et al., 2008; Chiang et al., 1998), and an electrocardiogram (ECG) signal was recorded with electrodes placed across the rat’s thorax.
3.2 Pulp exposure

The dental pulp of the right maxillary first molar tooth was exposed using a high-speed dental hand piece (Model D7950, Kavo, Germany) under occasional saline cooling while the rat lay in a supine position. The tooth was drilled until bleeding occurred, ensuring consistent pulp exposure and cavity depth. Thereafter, a saline-soaked cotton pellet covered the cavity until MO, MIN or PBS was applied.

3.3 Insertion of EMG electrodes

After an incision was made to expose the right and left anterior digastric (RAD, LAD) and masseter muscles, pairs of EMG electrodes, [40 gauge, 0.5-1 mm exposed tip, single stranded Teflon-insulated stainless steel wires, (Cooner wire, Chatsworth, CA, USA)] were implanted into the RAD and LAD (jaw-opening muscles), left and right masseter (jaw-closing muscles), trapezius muscle (shoulder-elevation muscle) and right forelimb muscle (the right extensor digitorum, a wrist-extensor muscle). A single EMG electrode was inserted into each of the left and right genioglossus (GG, tongue-protrusion) muscles. To confirm the adequate placement of the EMG electrodes within the muscles, and to ensure that the EMG electrodes were not displaced or that the muscles had not deteriorated during the experiment, a tetanic stimulation (12x0.2ms pulses, monophasic, monopolar cathodal 333 Hz) was applied to each muscle after EMG electrode placement and at the end of the experiment to produce evoked muscle twitches and ensure a stimulation threshold ≤ 200 µA.

3.4 Craniotomy and laminectomy

A craniotomy and cervical laminectomy were then performed to expose the left face-M1 and the caudal medulla, respectively (Fig. 2-2, 2-3). A stereotaxic apparatus (model 1340, David Kopf, Tujunga, CA, USA) was used to fix the rat’s head into a standardized position, whereby 2 ear rods coated with topical lidocaine (Xylocaine, AstraZeneca) were inserted into the ears’ left and right external meatus, a bite bar held the rat’s incisors in place, and horizontal bars carried the micropositioner for the ICMS electrode. A midline incision was made and bone wax was applied to the skull surface to prevent any bleeding. With bregma as the reference point, a dental hand piece at low-speed was used to cut through the skull to
expose the left hemisphere between anterior-posterior (AP) coordinates 0.0 and 5.0 mm rostral to Bregma and 1.0 mm to the most lateral point possible (~5.5 mm) mediolaterally (ML), thereby avoiding bleeding from the sagittal suture medially or damaging the orbital and temporal bones and their associated soft tissues. The dura around the AP 3 ML 3 stimulation site was removed to allow for the MSO or PBS application directly onto the cortical surface. Care was taken to minimize exposure of the cortex to air, which can lead to an inflammatory reaction and swelling (Shih et al., 2012). A ridge of vaseline was created around the exposed cortex and filled with mineral oil kept at 37 °C.

The caudal medulla was surgically exposed with a scalpel, and the neck muscles were separated with a retractor. The overlying dura and subarachnoid membrane were also removed with a scalpel and covered with a cotton pellet soaked in PBS, which was removed when MSO or PBS was applied to the caudal medulla.

![Fig. 2-2. Craniotomy exposing the left face-M1.](image-url)
Fig. 2-3. Cervical laminectomy exposing the caudal medulla. (Abbreviations: MSO – methionine sulfoximine, PBS – phosphate-buffered saline)

4. ICMS

4.1 The microelectrode

A glass-insulated tungsten microelectrode (10-20 µm exposed tip, 125 µm shank diameter and 300 µm outer diameter) (Alpha-Omega Engineering, Nazareth, Israel) was used for ICMS. Microelectrode impedance ranged from 1.0 – 3.0 MΩ and 250 – 450 pF. Low microelectrode impedance, such as used in this study, reduces cell damage, while high microelectrode impedance can result in a large electrode voltage and produce electrochemical reactions harmful to cells (Franks et al., 2005). A hooked rod was connected to the flap of skin overlying the cervical laminectomy as a ground for the stimulation.

4.2. Positioning of cortical stimulation site

The microelectrode was positioned with a micropositioner (Kopf model 2260) within the left face-M1 at a coordinate 3.0 mm anterior and 3.0 mm lateral to bregma (AP 3.0, ML 3.0), which was used as a reference point. Areas of the cortex with major blood vessels were not penetrated to avoid haemorrhaging. Based on previous studies around the location AP 3.0, ML 3.0 within the face-M1, ICMS can evoke EMG activity in the jaw or tongue muscles at a
low ICMS threshold (<30 µA) (Adachi et al., 2007, 2008; Avivi-Arber et al., 2010a, 2010b; Lee et al., 2006; Sapienza 1981).

4.3 ICMS-evoked EMG activity

The left face-M1 of rats contains representations of the RAD, LAD, and GG, and the neck and limb muscles are represented more medially. In preliminary experiments, EMG electrodes were implanted in all these muscles to determine if any of the muscle representations showed neuroplastic changes after MO application to the exposed tooth pulp. At AP 3.0 ML 3.0, the RAD was the most consistent muscle in which ICMS evoked RAD EMG activity at a low (30 µA) ICMS threshold intensity, and in which the changes in RAD threshold intensity displayed the most change after MO application to the exposed tooth pulp. Therefore, in subsequent experiments, analysis focused on the RAD. However, the other orofacial muscles were still implanted with EMG electrodes to maintain experimental consistency across groups and allow for future analysis of other muscles.

4.4 ICMS parameters

ICMS parameters (35ms train, 12x0.2ms pulses, 333Hz, ≤30µA) were consistent with previous studies in the laboratory (Adachi et al., 2007, 2008; Avivi-Arber et al., 2010a, 2010b; Huang et al., 1998). At the ICMS site, two trains of ICMS were delivered at 1-s intervals, initially at 60 µA. If this ICMS intensity evoked visible RAD EMG activity, the ICMS current was decreased in 10 µA steps. This was used to establish the current intensity at which no RAD EMG activity was evoked by ICMS; current was then progressively increased by 2 µA steps until the ICMS threshold intensity for evoking RAD EMG activity was determined on the basis of an ICMS-evoked response in at least one of the two stimulations at the lowest ICMS intensity. An ICMS site from which ICMS could evoke RAD EMG activity at an ICMS threshold intensity ≤30 µA was defined as a low-threshold site, and the series of ICMS trains from 60 µA to the lowest ICMS intensity (≤30 µA) was repeated after 15 minutes and after 30 minutes to establish the baseline ICMS threshold intensity.
The ICMS threshold intensity for evoking RAD EMG activity in face-M1 was only measured at depths between 1800 and 2400 µm, corresponding to cortical layers V and VI. Layer V of M1 contains pyramidal tract neurones, also named Betz cells, which are the main projection neurones to the brainstem motor nuclei and spinal cord, and therefore stimulation of layer V requires the lowest ICMS threshold intensity to evoke EMG muscle activity (Asanuma et al., 1976; Canedo, 1997; Mountcastle, 1994). Layer VI of M1 contains pyramidal tract neurones, as well as pyramidal cells that form corticocortical connections within M1 cortical columns, but also project to the thalamus (Thomson, 2010).

4.5 MO, MIN or PBS application to the molar tooth pulp

The cotton pellet in the tooth cavity was removed, and a thin piece of a dental paper point soaked in MO (allyl isothiocyanate, > 93%, Sigma Aldrich, USA) or vehicle control (MIN or PBS) was inserted into the cavity. Immediately following drug application, the cavity was then covered with a dental filling material (3M™ ESPE™ Cavit™) to prevent any leakage of MO, MIN or PBS.

4.6 MSO or PBS application to the medulla

In preliminary findings, the peak of the MO-induced increase in ICMS threshold intensity for evoking RAD EMG activity was determined to occur at 15 mins after MO application to the exposed tooth pulp (see Results). Therefore, at 15 mins following MO or vehicle application to the molar tooth pulp, 0.1 mM MSO (0.1 mM in PBS, Sigma Aldrich, USA) or PBS (Wisent Inc, Canada) heated to 37.5 °C in a water bath (Isotemp 102, Fisher Scientific) was applied via a 10 µL pipettor (Pipetman, Gilson, USA) to cover the exposed caudal medulla. Concentrations of MSO greater than 0.25 mM have been shown to depress neuronal function, while a concentration of 0.1 mM has been shown to be effective in attenuating MDH central sensitization (Chiang et al., 2007). During pilot experiments, varying concentrations of MSO (0.01 mM up to 1.0 mM) were applied to the exposed caudal medulla to test for effects on MO-induced increases in RAD thresholds, and it was determined that 0.1 mM was the optimal concentration to modify MO-induced increases in RAD thresholds and this concentration was used in subsequent experiments. PBS was used rather than Ringer’s solution to maintain cortical pH at neutral 7.4 (Dempsey et al., 1979). After MSO or
PBS application to the medulla, RAD threshold was measured immediately and then every 10 mins for the 120 mins-observation period.

4.7 Electrolytic lesion

At the termination of each experiment, an electrolytic lesion was placed by passing cathodal direct current (DC, 10 µA for 7 sec) at a depth of 3500 µm within the positive ICMS penetration site. Consistent with previous ICMS studies (Adachi et al., 2007; Avivi-Arber et al., 2011; Iriki et al., 1991; Toda and Taoka, 2004), this lesion was used for subsequent histological confirmation of the location of the ICMS site.

5. Histological procedures and verification of ICMS sites

After each ICMS experiment, the rat was deeply anaesthetized by an overdose of ketamine HCL and perfused trans-cardially with PBS, and then again with 10 % buffered formalin (Fisher Scientific, New Jersey, USA). The brain was then extracted and stored in 10 % buffered formalin. 100 µm thick coronal brain sections were sliced with a vibratome (Model 3000, TPI, Missouri, USA). Sections were stained with Nissl stain (Cresyl violet). Nissl stain is an aniline stain that reacts with nucleic acids (DNA, RNA) of neurones, revealing the cellular structure of the cortex (Kadar et al., 2009).

Histological sections (along with a ruler for calibration) were then scanned into a computer with a flatbed scanner with a resolution of 1200 dots per inch. Thereafter, the images were used to measure the location of the electrolytic lesions. Public domain Image-J software program (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2006) allowed visualization and measurement of vertical and mediolateral positions of the electrolytic lesions from the cortical surface and the midline, respectively. This measurement was used to correct the initial location recorded with the micropositioner-micromanipulator. The histological sections were then used to confirm that the low-threshold ICMS site for evoking EMG activity was within the M1 agranular cortex, which lacks the cytoarchitectonic granular layer IV of S1, and thus that ICMS was performed in the cortex and not in subcortical structures, and that no lesion was created by ICMS itself. M1 is located medial and rostral to S1. The Swanson atlas (Swanson,
2004) helped to identify anatomical landmarks and confirm that the ICMS penetration sites were in the vicinity of the cortical region defined as face-M1 at AP 3.0 ML 3.0. Any animal where the penetration was outside the agranular cortex was excluded from the data analysis.

6. Data acquisition and analysis

EMG responses were amplified using a gain of x1000 and filtered (bandpass 100~1 kHz) by an alternating current (AC) amplifier (A-M system, Washington, USA, model 1700). The signals were digitized at 5 kHz by an analog to digital converter (CED 1401 plus, Cambridge Electronic Design, Cambridge, UK), which was performed on a personal computer. Customized software allowed analysis of data files off-line (Adachi et al., 2007; Avivi-Arber et al., 2010). Programs were written in Spike2 script (CED, Cambridge Electronic Design, Cambridge, UK) and LabView (National Instruments, Austin, TX, USA). For the RAD at each ICMS site, the 2 ICMS-evoked EMG waveforms, corresponding to the series of 2 stimulation trains, were rectified, averaged and smoothed by a 4-msec moving-average window (Fig. 2-4a, 2-4b) (Baker and Lemon, 1995; Myers et al., 2003; for review, see Cheney, 2002). The Spike2 script separated EMG waveforms into 100 msec data segments, allowing the LabView program to determine the EMG waveform parameters. The programs were then used to analyze the threshold and onset latency parameters of ICMS-evoked EMG activities, where onset latency was defined as the time between the start of the stimulation train and the time when the averaged ICMS-evoked response exceeded 2 standard deviations (SD) of the mean value of the initial 10 msec of the EMG activity immediately following the start of the ICMS train.

The computer algorithm analyzed ICMS-evoked RAD EMG activity by 2 criteria to identify ICMS-evoked RAD EMG activity (Fig. 2-5):

1. at least one of the two ICMS trials at ICMS threshold intensity evoked EMG activity (Hodges and Bui, 1996). Visual identification of EMG activity was also performed during recording, as the customized software did not always detect movement artifacts or ECG activity artifacts that could overlap with EMG activity (Hodges and Bui, 1996),
2. after rectifying, averaging and smoothing raw EMG activity, peak EMG activity exceeded the mean value of the initial 10 msec baseline EMG activity immediately following the start of the ICMS train by 2 SD (95% confidence interval).

**Fig. 2-4a.** Suprathreshold ICMS-evoked EMG response rectified, averaged and smoothed by a 4msec-moving average window. 0 sec corresponds to start of stimulus train.
**Fig. 2-4b.** Threshold ICMS-evoked EMG activity rectified, averaged and smoothed by a 4 msec-moving average window. 0 seconds corresponds to start of stimulus train.

**Fig. 2-5.** ICMS-evoked EMG activity recorded from the RAD muscle.

7. **Statistical analyses**

7.1 **Sample size calculation**

To estimate the number of rats required in each experimental or control group, the following sample size calculation was performed:

Using the formula to compare two means:

\[
\frac{(u+v)^2}{\sigma_1^2 + \sigma_0^2} \cdot (\mu_1 - \mu_2)
\]

(Kirkwood and Sterne, 2003)

where:

- \(u\) – one sided percentage point of the normal distribution corresponding to 100 % - the power
- \(v\) – percentage point of the normal distribution corresponding to the (two-sided) significance level
- \(\sigma_1, \sigma_2\) – standard deviations
- \(\mu_1 - \mu_2\) – difference between means

The number of rats required in each group to obtain 95% power (\(u=1.64\)), significant at a level of 5% (\(v=1.96\)) with a 45% difference between means (\(\mu_1 - \mu_2=0.45\)), and a standard deviation of 25% in each group (\(\sigma_1 = \sigma_2 = 0.25\)), is:

\[
\frac{(1.64+1.96)^2}{(0.25^2+0.25^2)}
\]
\[(0.45)^2\]

\[= 8\]

For 5 groups and subgroups (MO/PBS, MO/MSO, vehicle/PBS, vehicle/MSO, naïve), with 8 rats/group, 40 rats were needed to detect significant differences between study groups and subgroups.

### 7.2. Analysis of Significance

SigmaPlot 11 (Systat Software, San Jose, CA, USA) was used to perform statistical analysis. The first series of data analyses used repeated-measures (RM) ANOVA followed by *post-hoc* Bonferroni-adjusted pairwise comparisons as appropriate to test within each study group (MO, vehicle, MO/PBS, MO/MSO, vehicle/PBS, vehicle/MSO, naïve) the effect of chemical application on RAD thresholds and onset latencies over time (i.e., baseline, 1, 5 and 15 mins after MO or vehicle application to the molar tooth pulp, and 15, 30, 60 and 120 mins after MSO or PBS application to the medulla). In the second set of data analyses, at each of the studied time points, one-way ANOVA followed by *post-hoc* Bonferroni-adjusted pairwise comparisons as appropriate was used to compare the RAD thresholds and onset latencies across all study groups. All values are reported as mean ± SEM. In all analyses a probability level of \(p<0.05\) was considered statistically significant.
Chapter 3

Results

1. Histological verification of ICMS sites

Measurements made with Image-J software revealed the sites of electrolytic lesions and confirmed ICMS sites were located at depths ranging between 1.8 and 2.5 mm and coinciding with the cytoarchitectonically defined layers V-VI of the agranular cortex at AP coordinate 3.0 ± 0.5 mm and ML coordinates 3.0 ± 0.5 mm (Fig. 3-1a), coinciding with the face-M1 region (Swanson, 2004). Histological analysis also revealed no evidence of cortical tissue damage as a result of the repeated ICMS that was required for the repeated determination of the ICMS threshold intensities for evoking RAD EMG activities during the 2-4 hours of ICMS.

**Fig. 3-1a.** Histological coronal section at AP 3.0 anterior to bregma illustrating the location of the primary motor cortex (M1) within the agranular cortex and the primary somatosensory cortex (S1) within the granular cortex at AP 3.0. Bottom of arrowhead indicates the electrolytic lesion, while numbers indicate the cytoarchitectonic layers.

**b.** A schematic diagram of a rat coronal section at AP 3.0 anterior to bregma indicating the layers of the sensorimotor cortex and the borders between the M1 (agranular), S1 (granular). (M1: primary motor cortex, S1: primary somatosensory cortex (Adapted from Swanson atlas (Swanson, 2004)).
2. Baseline parameters of RAD EMG activity and ICMS-evoked RAD EMG activity

2.1. RAD EMG activity

Prior to the ICMS experiments, baseline RAD EMG activity was monitored for 30 mins and showed a stable background activity with at least 4-8 spontaneous EMG bursts per minute (Fig. 3-2), indicating an adequate state of general anaesthesia.

![Fig. 3-2](image)

Fig. 3-2. Baseline RAD EMG activity showing 5 spontaneous EMG bursts per minute.

(Abbreviations: ▼- spontaneous EMG bursts, RAD – right anterior digastric muscle).

2.2. ICMS threshold intensities

At baseline, in all rats, ICMS applied within the left face-M1 could evoke RAD EMG activities at a low ICMS threshold intensity ranging between 17-30 µA (24.9 ± 0.8 µA). In the experimental and control groups, these RAD thresholds were found to be stable during the 30-minute baseline recordings with no significant differences across study groups (ANOVA, F(2,37)=4.7, p=0.1) (Table 3-1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline Threshold (Mean ± SEM) (µA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO (n=16)</td>
<td>22.4 ± 1.1</td>
</tr>
<tr>
<td>Vehicle (n=16)</td>
<td>25.9 ± 1.1</td>
</tr>
<tr>
<td>Naïve (n=8)</td>
<td>27.8 ± 1.4</td>
</tr>
</tbody>
</table>

Table 3-1. Baseline mean ICMS threshold intensities for evoking RAD EMG activity in the MO, vehicle and naïve groups. There were no significant differences across the study groups.

2.3. EMG onset latencies

During the 30 mins of baseline recording, the mean onset latencies of RAD EMG activity evoked by 60 µA ICMS were stable and ranged from 8.0-29.2 msec in all rats (mean 15.3 ± 1.5 msec). There were no significant differences in the baseline mean onset latencies across study groups (ANOVA, F(2,37)=2.6, p = 0.1) (Tables 3-2).

<table>
<thead>
<tr>
<th>Group</th>
<th>Onset Latency (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± SEM)</td>
</tr>
<tr>
<td>MO (n=16)</td>
<td>12.4 ± 1.0</td>
</tr>
<tr>
<td>Vehicle (n=16)</td>
<td>15.0 ± 2.5</td>
</tr>
<tr>
<td>Naïve (n=8)</td>
<td>21.1 ± 4.7</td>
</tr>
</tbody>
</table>

Table 3-2. Baseline mean onset latencies of ICMS-evoked RAD EMG activities evoked by 60 µA ICMS across MO, vehicle and naïve groups. There were no significant differences in baseline mean RAD onset latencies across the study groups (Abbreviations: EMG – electromyographic, ICMS – intracortical microstimulation, MO – mustard oil, PBS – phosphate buffered, RAD – right anterior digastric muscle).

3. Effects of MIN versus PBS application to the molar tooth pulp

3.1. RAD EMG activity

PBS and MIN were applied to the molar tooth pulp as a control for the inflammatory irritant MO. Neither PBS nor MIN application to the molar tooth pulp had any apparent effect on RAD EMG activity (Fig. 3-3B).
Fig. 3-3. RAD EMG recordings in a representative rat from the MIN group. (A) ICMS-evoked RAD EMG activity recorded at baseline, showing baseline ICMS threshold intensity of 26 µA for evoking RAD EMG activity. (B) RAD EMG activity showing no change immediately following MIN application to the tooth pulp. In addition, RAD threshold immediately following MIN application to the tooth pulp was similar to baseline at 26 µA. (C) RAD EMG activity 15 mins after MIN application to the molar tooth pulp, with RAD threshold similar to baseline at 26 µA.  (* - ICMS-evoked EMG activity, ▼ - spontaneous EMG activity. Abbreviations: EMG – electromyographic, ICMS – intracortical microstimulation, MIN – mineral oil, PBS – phosphate-buffered saline, RAD – right anterior digastric muscle).

3.2. ICMS threshold intensities

Neither MIN nor PBS had any significant effects 1, 5 or 15 mins after application to the molar tooth pulp on ICMS threshold intensities for evoking RAD EMG activities compared to baseline (MIN: RM ANOVA, F(2,14)=0.3, p=0.7; PBS: RM ANOVA, F(2,14)=0.8, p=0.5). There were also no significant differences in RAD thresholds
between PBS and MIN groups at the same time points (ANOVA, $F_{(2,21)} = 0.7$, $p=0.5$; $F_{(2,21)} = 0.6$, $p=0.6$; $F_{(2,21)} = 1.4$, $p=0.3$, respectively)(Figs. 3-3B, C; 3-4; Table 3-3).

**Fig. 3-4.** This graph shows the mean percent change in ICMS threshold intensity for evoking RAD EMG activity 1, 5 and 15 mins following PBS or MIN application to the molar tooth pulp. Neither PBS nor MIN had an effect on baseline RAD threshold (Abbreviations: EMG – electromyographic, ICMS – intracortical microstimulation, MIN – mineral oil; PBS – phosphate-buffered saline, RAD – right anterior digastric muscle).

### 3.3. EMG onset latencies

In comparison to baseline values, neither PBS nor MIN application to the molar tooth pulp had a significant effect on the mean onset latencies of ICMS-evoked RAD EMG activity (at 60 μA ICMS) (MIN: RM ANOVA, $F_{(3,20)} = 1.2$, $p=0.4$; PBS: RM ANOVA, $F_{(3,20)} = 1.6$, $p=0.2$; Table 3-3).
<table>
<thead>
<tr>
<th>Group</th>
<th>Time after application to the pulp</th>
<th>Threshold (µA) (Mean ± SEM)</th>
<th>Onset Latency (msec) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS to pulp</td>
<td>Baseline</td>
<td>23.3 ± 1.0</td>
<td>13.7 ± 2.3</td>
</tr>
<tr>
<td>(n=8)</td>
<td>1 min</td>
<td>23.3 ± 1.1</td>
<td>11.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>5 mins</td>
<td>23.5 ± 1.0</td>
<td>11.3 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>15 mins</td>
<td>23.1 ± 1.0</td>
<td>15.6 ± 2.8</td>
</tr>
<tr>
<td>MIN to pulp</td>
<td>Baseline</td>
<td>28.5 ± 1.4</td>
<td>16.2 ± 4.3</td>
</tr>
<tr>
<td>(n=8)</td>
<td>1 min</td>
<td>27.7 ± 1.0</td>
<td>19.8 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>5 mins</td>
<td>28.0 ± 1.0</td>
<td>14.5 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>15 mins</td>
<td>27.6 ± 1.4</td>
<td>19.8 ± 4.6</td>
</tr>
</tbody>
</table>

Table 3-3. Mean ICMS threshold intensities for evoking RAD EMG activity and mean onset latencies of RAD EMG activity evoked by 60 µA ICMS before, and at 1, 5 and 15 mins following application of PBS or MIN to the molar tooth pulp. There were no statistically significant effects of PBS or MIN on the thresholds or onset latencies, and no differences in the mean RAD threshold intensities or mean onset latencies between PBS and MIN application to the molar tooth pulp (Abbreviations: EMG – electromyographic, ICMS – intracortical microstimulation, MIN – mineral oil, PBS – phosphate-buffered saline, RAD – right anterior digastric muscle).

Since there were no significant differences between PBS and MIN application to the molar tooth pulp on the RAD EMG activities, thresholds and onset latencies, the data from these 2 groups were pooled into one ‘vehicle group’.

53
4. Naïve group

4.1. ICMS threshold intensities

In naïve rats, ICMS threshold intensities for evoking RAD EMG activities remained stable for at least 120 mins, with no significant changes over time as compared to baseline values (RM ANOVA, $F_{(7,48)}=1.7$, $p=0.1$) (Fig. 3-7). There were also no significant differences in the mean RAD thresholds between the naïve and vehicle/PBS control group at any of the studied time points (e.g., 1, 5, 15 min post MO application, 1, 15, 30, 60 min post MSO application) (ANOVA: MO: $F_{(1,22)}=0.1$, 5.0, 5.0, $p=0.8$, 0.8, 0.8; MSO: $F_{(1,14)}=1.9$, 2.4, 0.0, $p=0.2$, 0.1, 0.9, respectively) (Fig. 3-7).

4.2. EMG onset latencies

In naïve rats, mean onset latencies for evoking RAD EMG activities at 60 $\mu$A ICMS could also be stable for at least 120 mins (RM ANOVA, $F_{(7,48)}=1.1$, $p=0.4$) (Table 3-4). There were no significant differences in the mean onset latencies between the naïve and vehicle/PBS control group at any of the studied time points (ANOVA, $F_{(1,21)}=1.6$, 2.0, 2.2, 2.4; $p=0.2$, 0.2, 0.2, 0.1; $F_{(1,13)}=2.9$, 2.9, 0.6, 5.1; $p=0.1$, 0.1, 0.5, 0.1, respectively).

5. Effects of MO application to the molar tooth pulp on RAD EMG activity and ICMS parameters

5.1. RAD EMG activity

MO application to the molar tooth pulp resulted in a transient increase in RAD EMG activity that lasted up to 1 minute (Fig. 3-5B).
Fig. 3-5. (A) EMG activity recorded from the RAD at baseline, with baseline ICMS threshold intensity measured as 17 µA. (B) EMG activity of the RAD showing increased activity immediately after MO application to the molar tooth pulp. RAD threshold can be seen to have increased to 24 µA. (C) EMG activity of the RAD 15 mins after MO application to the molar tooth pulp showing further increase in RAD threshold to 30 µA. (Abbreviations: * - ICMS-evoked EMG activity, ▼ - spontaneous EMG bursts, EMG – electromyographic, ICMS – intracortical microstimulation, MO – mustard oil, RAD – right anterior digastric muscle).
5.2. ICMS threshold intensities

In comparison with baseline, ICMS threshold intensities for evoking RAD EMG activities were significantly increased after MO application to the molar tooth pulp. At the measured 1, 5 and 15 mins time points following MO application to the molar tooth pulp, there was an increase in RAD thresholds of $26.3 \pm 10.2\%$, $35.0 \pm 8.2\%$, and $46.3 \pm 7.9\%$, respectively (RM ANOVA, $F_{(3,48)}=13.5$, $p<0.001$, followed by post-hoc Bonferroni, $p=0.003$; $p<0.001$; $p<0.001$, respectively) (Fig. 3-7). Similarly, in comparison to vehicle application to the molar tooth pulp, RAD thresholds were significantly increased 1, 5 and 15 mins following MO application to the tooth pulp by $23.5 \pm 10.2\%$, $31.0 \pm 8.2\%$, and $44.0 \pm 7.2\%$, respectively (ANOVA, $F_{(1,31)}=6.3$, $p<0.02$, followed by post-hoc Bonferroni, $p=0.02$; $F_{(1,31)}=16.8$, $p<0.001$, followed by post-hoc Bonferroni, $p<0.001$; $F_{(1,31)}=33.8$, $p<0.001$, followed by post-hoc Bonferroni, $p<0.001$, respectively) (Fig. 3-7).

5.3. EMG onset latencies

There were no significant differences between the mean onset latencies for evoking RAD EMG activities at 60 $\mu$A ICMS at any time point after MO application to the molar tooth pulp compared to baseline (RM ANOVA, $F_{(3,37)}=1.4$, $p=0.1$) (Table 3-4).

6. Effects of MSO or PBS application to the medulla on the MO-induced changes in RAD EMG activity and ICMS parameters

6.1. RAD EMG activity

Neither MSO nor PBS application to the medulla had any effect on RAD EMG activities (Fig. 3-6).
Fig. 3-6. 15 mins after MSO application to the medulla, EMG activity of the RAD is similar to baseline. (Abbreviations: * - ICMS-evoked EMG activity, ▼ - spontaneous EMG bursts, EMG – electromyographic, ICMS – intracortical microstimulation, MSO – methionine sulfoximine, RAD – right anterior digastric muscle).

6.2. ICMS threshold intensities

Application to the medulla of PBS (MO/PBS group) had no significant effect 15, 30, or 60 mins later on the MO-induced increase in ICMS threshold intensities for evoking RAD EMG activities, since the ICMS threshold intensities remained increased compared to the MO-induced RAD thresholds (MO group) (RM ANOVA, F(3,17)=0.9, p=0.5, 37.9%±6.0%, 28.1%±2.7%, 29.5%±4.3%, respectively). These thresholds also remained significantly increased compared to baseline, with increases of 37.9%±6.0% at 15 mins, 28.1%±2.7% at 30 mins and 29.5%±4.3% at 60 mins following PBS application to the medulla (MO/PBS group) (RM ANOVA, F(3,17)=31.8, p<0.001, followed by post-hoc Bonferroni, p<0.001, p<0.001, p<0.001, respectively). Compared to the same time points in the naïve group, RAD thresholds were significantly increased at 15, 30 and 60 mins after PBS application to the medulla (MO/PBS group) (ANOVA, F(1,13)=14.6, p=0.002, followed by Bonferroni, p=0.002; 33.8±3.8%, F(1,13)=10.2, p=0.01, followed by post-hoc Bonferroni, p=0.01; 16.8±3.2%, F(1,13)=8.2, p=0.01, followed by post-hoc Bonferroni, p=0.01, 20.7±2.9%)(Fig. 3-7). At 120 mins after application of PBS to the medulla (MO/PBS group), RAD thresholds had decreased and were not significantly different from the
control (vehicle/PBS) group at the same time point (ANOVA, F(1,8)=2.2, p=0.2) (Fig. 3-7).

In contrast with the lack of PBS effects, application of MSO to the medulla (MO/MSO group) resulted, at 15, 30 and 60 mins later, in significant decreases in RAD thresholds compared to the MO-induced RAD thresholds of 27.5±3.0%, 27.2±3.0%, and 26.5±3.0%, respectively (RM ANOVA, F(3,18)=4.7, p=0.01, followed by post-hoc Bonferroni p=0.05, p=0.04, p=0.05, respectively) (Fig. 3-7). In comparison with RAD thresholds following PBS application to the medulla (MO/PBS group), RAD thresholds were decreased at 15, 30, and 60 mins time points following MSO application to the medulla by 26.1±2.7%, 12.3±1.8% and 23.2±4.7%, respectively (ANOVA, F(1,13)=4.7, p=0.05, followed by post-hoc Bonferroni, p=0.05; F(1,13)= 2.2, p=0.2; F(1,13)= 22.4, p<0.001, followed by post-hoc Bonferroni, p<0.001, respectively) (Fig. 3-7). At 120 mins following MSO application to the medulla, RAD thresholds were significantly decreased compared to PBS application to the medulla (MO/PBS group) by 35.5±4.5% (ANOVA, F(1,13)=5.7, p=0.005, followed by post-hoc Bonferroni, p=0.004).

As mentioned above, vehicle application to the tooth pulp had no effect on RAD thresholds. Subsequent (15 mins later) application of MSO or PBS to the medulla also had no significant effects on RAD thresholds, which remained comparable to baseline values 15, 30, 60 and 120 mins after MSO or PBS application to the medulla (RM ANOVA, vehicle/MSO group: F(4,22)=2.6, p=0.1, vehicle/PBS group: F(4,22)=1.7, p=0.2)(Fig.3-7).

6.3. EMG onset latencies

Neither MSO nor PBS application to the medulla following either MO (MO/MSO, MO/PBS groups) or vehicle (vehicle/MSO, vehicle/PBS groups) application to the molar tooth pulp had any significant effects on the onset latencies of RAD EMG
activities evoked by 60 µA ICMS, which remained comparable to baseline values at 15, 30, 60 and 120 mins later (RM ANOVA, MO/MSO group: $F_{(4,22)}=0.6$, $p=0.7$; MO/PBS group: $F_{(4,18)}=1.1$, $p=0.4$; vehicle/MSO group: $F_{(4,25)}=3.7$, $p=0.2$; vehicle/PBS group: $F_{(4,23)}=1.5$, $p=0.2$) (Table 3-4). As well, there were no significant differences in onset latencies across the study groups at any of the 15, 30, 60 and 120 mins time points (ANOVA, $F_{(3,26)}=1.0$, $p=0.4$; $F_{(3,26)}=1.0$, $p=0.4$; $F_{(3,26)}=2.2$, $p=0.1$; $F_{(3,13)}=0.8$, $p=0.5$; respectively)(Table 3-4).

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (MO or vehicle) (msec) (Mean ± SEM)</th>
<th>1 min (msec) (Mean ± SEM)</th>
<th>5 mins (msec) (Mean ± SEM)</th>
<th>15 mins (MO or PBS) (msec) (Mean ± SEM)</th>
<th>30 mins (msec) (Mean ± SEM)</th>
<th>60 mins (msec) (Mean ± SEM)</th>
<th>120 mins (msec) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO/MSO</td>
<td>12.4 ± 1.0</td>
<td>11.2 ± 0.7</td>
<td>17.7 ± 2.9</td>
<td>14.1 ± 2.6</td>
<td>11.8 ± 0.8</td>
<td>11.8 ± 0.8</td>
<td>12.3 ± 2.5</td>
</tr>
<tr>
<td>MO/PBS</td>
<td>12.4 ± 1.0</td>
<td>11.2 ± 0.7</td>
<td>17.7 ± 2.9</td>
<td>14.1 ± 2.6</td>
<td>12.0 ± 1.6</td>
<td>12.0 ± 1.6</td>
<td>11.7 ± 1.1</td>
</tr>
<tr>
<td>Vehicle/PBS</td>
<td>15.0 ± 2.5</td>
<td>15.3 ± 1.8</td>
<td>12.9 ± 1.4</td>
<td>17.7 ± 2.7</td>
<td>16.0 ± 3.3</td>
<td>16.0 ± 3.3</td>
<td>21.6 ± 5.0</td>
</tr>
<tr>
<td>Vehicle/MSO</td>
<td>15.0 ± 2.5</td>
<td>15.3 ± 1.8</td>
<td>12.9 ± 1.4</td>
<td>17.7 ± 2.7</td>
<td>12.7 ± 1.0</td>
<td>12.7 ± 1.0</td>
<td>12.3 ± 1.1</td>
</tr>
<tr>
<td>Naïve</td>
<td>21.1 ± 4.7</td>
<td>21.7 ± 5.6</td>
<td>18.9 ± 5.8</td>
<td>25.7 ± 5.0</td>
<td>25.8 ± 4.9</td>
<td>25.8 ± 4.9</td>
<td>26.5 ± 3.6</td>
</tr>
</tbody>
</table>

**Table 3-4.** Mean onset latencies of ICMS-evoked RAD EMG activities evoked by 60 µA ICMS across study groups at all analyzed time points. (Abbreviations: MO – mustard oil, PBS – phosphate-buffered saline).
Fig. 3-7. The percent change from baseline in ICMS threshold intensity for evoking RAD EMG activity across all study groups. Error bars represent SEM.

*  p<0.05 compared to baseline (RM ANOVA, post-hoc Bonferroni)
** p<0.001 compared to baseline (RM ANOVA, post-hoc Bonferroni)
°  p<0.05 compared to vehicle/vehicle (RM ANOVA, post-hoc Bonferroni)
° ° p<0.02 compared to vehicle/vehicle (RM ANOVA, post-hoc Bonferroni)
•  p<0.05 compared to MO/MSO (RM ANOVA, post-hoc Bonferroni)

Chapter 4

Discussion

Using the acute inflammatory dental pain model, this study has shown that MO (but not vehicle control) application to the rat molar tooth pulp resulted in an immediate but transient (12-60 secs) increase in RAD EMG activity. There was also a decreased excitability of the contralateral face-M1 manifested as an increased ICMS threshold intensity for evoking RAD EMG activity that peaked 15 mins after MO application to the molar tooth pulp. The study also demonstrated for the first time that topical application of the astrocyte inhibitor MSO (but not vehicle control) to the caudal medulla reversed, within 15 mins, the MO-induced change in face-M1 excitability that was restored to baseline levels within 60 mins. In contrast to the changes observed in RAD threshold, the onset latencies of RAD EMG activities evoked by 60 µA (suprathreshold) ICMS were not affected by either MO (or vehicle control) application to the molar tooth pulp or MSO (or PBS control) application to the caudal medulla. It has been previously documented that MO application to the rat molar tooth pulp is associated with increased excitability (central sensitization) of nociceptive neurones within the MDH in the caudal medulla and that MSO application to the caudal medulla can prevent or reverse the MO-induced MDH central sensitization (Chiang et al., 2007, 2011, 2012). Therefore, our novel findings suggest that medullary nociceptive neurones play a crucial role in transmitting nociceptive information to the face-M1 that can modulate face-M1 excitability, and that this mechanism involves medullary astrocytes. These findings may have clinical implications as they may be related to the mechanisms by which acute pain in humans is associated with limited jaw movements, and they also suggest medullary astrocytes may offer novel therapeutic targets for the prevention and treatment of acute dental pain and impaired motor function.

1. General features of ICMS-evoked RAD EMG activity

Histological analysis revealed that in all study groups, the studied ICMS sites were located within layers V-VI of the cytoarchitectonically defined granular cortex coinciding with face-M1 at coronal plane 3.0 mm anterior to bregma (Swanson, 2004). Consistent with previously
published studies, at baseline, short-train ICMS evoked RAD EMG activities at low (<30 µA) ICMS threshold intensities and that at suprathreshold ICMS (60 µA), the evoked activities had onset latencies ranging between 8-29.2 msec (Adachi et al., 2008; Avivi-Arber et al., 2010a, b; Donoghue and Wise, 1982; Neafsey et al., 1986). In addition, onset latencies at RAD threshold intensities were longer than at 60 µA ICMS and ranged between 12-40 msec. Onset latency is a reflection of several factors including conduction velocity, distance and number of synapses along the corticomotor pathways to the RAD motoneurones. Conduction velocity depends on (but is not limited to) the diameter and myelination of the axon (Chapman and Yeomans, 1994; Tehovnik et al., 2006). Increased ICMS intensity is associated with an increased current spread (Asanuma 1989; Tehovnik et al., 2006). Our findings of a range of RAD onset latencies, with decreased onset latency associated with an increase in ICMS intensity suggest that RAD may be represented within a number of spatially related face-M1 loci from which projection neurones to RAD motoneurones vary in their diameter, myelination and number of synapses (Schieber 2001; Tehnovik et al., 2006).

There were no significant differences and no changes over time in RAD thresholds or onset latencies between the naïve group and the vehicle control group that received all experimental procedures (tooth drilling, cervical laminectomy, craniotomy, ICMS) but only vehicle control applications. This suggests that the experimental procedures did not have a significant effect on the studied outcome measures.

2. Acute inflammatory dental pain model

MO is a small-fibre excitant and inflammatory irritant that can directly activate the TRPA1 receptor, which is present on nociceptive primary afferent endings (including dental pulp afferents) (Kim et al., 2012), or it can activate TRPA1 receptors indirectly through the release of the inflammatory mediator BK (Bautista, 2013). This results in a barrage of nociceptive inputs from nociceptive primary afferents to nociceptive neurones of the VBSNC. TRPA1 expression in rodent tooth pulps has been shown to increase after tooth injury (Haas et al., 2011). Therefore, MO application to the tooth pulp has been utilized in the present and other studies as a model of acute inflammatory dental pain to study the mechanisms underlying peripheral and central trigeminal nociceptive processing (Chiang et
al., 1998, 2002, 2005, 2007, 2010; Narita et al., 2012; Sunakawa et al., 1999; Xie et al., 2007; Zhang et al., 2006). It has been shown that noxious stimulation by MO application to the rat maxillary molar tooth pulp at the dose (0.1mM) used in the present study, as well as other orofacial noxious stimuli, can induce neuroplastic changes reflected in peripheral sensitization (increased excitability) of trigeminal nociceptors (Sessle, 2011b) as well as central sensitization of nociceptive neurones within the MDH (Chiang et al., 2007, Xie et al., 2007, Zhang et al., 2006), thalamus (Katz et al., 1999; Kawamura et al., 2010; Park et al., 2006; Zhang et al., 2006) and face-S1 (Calford and Tweedale, 1991; de Leeuw et al., 2006; Jantsch et al., 2005; Kupers et al., 2004; Weigelt et al., 2010). Peripheral and central sensitization can be manifested as increased neuronal responses to innocuous and noxious stimuli, lowered activations thresholds, increased spontaneous activity and expanded neuronal RFs. Such changes can contribute to the clinical manifestations of primary hyperalgesia, allodynia, spontaneous pain and pain spread or referral associated with acute (and chronic) orofacial pain conditions (Chiang et al., 2012; Latremoliere and Woolf, 2009; Sessle, 2011b).

2.1. Effects of MO molar tooth pulp application on RAD EMG activity

Consistent with previous studies in our laboratory, acute noxious stimulation induced by MO application to the rat molar tooth pulp resulted in a transient increase in EMG activity in the RAD that lasted for up to 1 min (Narita et al., 2012; Sunakawa et al., 1999). This reflexive and transient increase in RAD EMG activity is also consistent with studies applying noxious stimulation to other orofacial tissues (e.g., TMJ)(Cairns et al., 2001b; Tsai et al., 1999; Yu et al., 1995). Such increased muscle activity may have clinical implications as it may facilitate nocifensive escape behaviour to protect the injured tissue.

2.2. Effects on Face-M1 excitability

The novel finding from the present study indicates that application of MO (but not vehicle control) to the rat molar tooth pulp rapidly (within 15 mins) leads to increases in the ICMS threshold intensity for evoking RAD EMG activities that can last up to 120 mins. An
increase in RAD threshold following MO application to the molar tooth pulp implies a
decreased face-M1 excitability (Adachi et al., 2008). However, since ICMS of face-M1
evokes EMG activities through activation of brainstem motoneurones, which represent the
final common integrating outputs for a large number of sensory and motor inputs projecting
to the orofacial muscles (Capra, 1995; Paxinos, 2004; Sessle, 2000; Trulsson and Essick,
2004), it is unclear whether the observed changes in face-M1 excitability are the result solely
of changes within the face-M1 or whether they also reflect changes at subcortical levels such
as the Vm.

A novel finding of this study includes the demonstration that the rapid (within 15 mins)
increase in face-M1 excitability following MO application to the rat molar tooth pulp can be
rapidly (within 15 mins) attenuated by subsequent application to the medulla of MSO, an
inhibitor of the astrocytic enzyme GS. These findings suggest that medullary astrocytes are
involved, at least in part, in modulating face-M1 excitability. However, the exact brainstem
mechanisms are still unclear, and whether face-M1 mechanisms are also involved has not
been fully elucidated, although there is recent evidence that MSO application to the face-M1
can reverse the decreased face-M1 excitability following MO application to the rat molar
tooth pulp (Awamleh et al., 2014). Similarly, it is also unclear whether medullary astrocytes
are involved in modulating nociceptive transmission to face-S1 or Vm.

2.2.1. Findings in related studies

This project’s findings of a noxious stimulus-induced transient increase in RAD EMG
activity and a decrease in face-M1 excitability are consistent with other studies in animals
and humans whereby experimental acute noxious stimulation were applied to other orofacial
tissues. For example, in rats, injection of MO into the molar tooth pulp or the
temporomandibular joint evokes a transient increase in jaw muscle EMG activity (Narita et
al., 2012; Sunakawa et al., 1999; Tsai et al., 1999; Yu et al., 1995), while in humans, this
finding can be replicated with application of hypertonic saline to the masseter muscle
(Svensson et al., 1997). Injection of glutamate into the rat tongue (Adachi et al., 2008) or
capsaicin application to the tongue in humans (Boudreau et al., 2007) is associated with
decreased face-M1 excitability as defined by ICMS or TMS, respectively. Injection of
hypertonic saline into the masseter muscle in humans results in an initial increased activation followed by a decreased activation of face-M1 as defined by fMRI (Nash et al., 2010). These findings may have clinical significance in pointing to the involvement of face-M1 in early as well as late pain-related alterations in orofacial motor functions. It can be speculated that the initial increases in muscle EMG activity or fMRI-defined M1 activity following noxious orofacial stimulation may underlie a nocifensive escape-like motor behaviour. However, consistent with the ‘Pain Adaptation Model’ (Lund et al., 1991; Murray and Peck, 2007), once the initial threat subsides, longer-term reductions in M1 activity may occur and contribute to the clinical manifestation of limited jaw movement to prevent further tissue damage in subjects suffering from acute (and chronic) orofacial pain (Lund et al., 1991; Murray and Peck, 2007; Nash et al., 2010; Stohler and Zarb, 1999; Svensson and Graven-Nielsen, 2001). The increased jaw EMG activity induced by noxious stimulation to the tooth pulp may be related to trigeminal central sensitization, as studies have shown that the reflex jaw responses can be attenuated by MDH transection, blocking of MDH with NMDA receptor antagonists or local anaesthetic (Cairns et al., 1998, 2001b; Kumar et al., 2012; Tsai et al., 1999), or by inhibiting glutamate release from the MDH with the drug pregabalin (Kumar et al., 2012; Narita et al., 2012); these manipulations also attenuate MDH central sensitization. As acute pain progresses into chronic pain, there may be a loss of local inhibitory GABAergic interneuronal activity in M1 as well as in the MDH and other CNS pain-processing areas (e.g. thalamic reticular nucleus), such as found in neuropathic pain (Henderson et al., 2013; Schabrun and Hodges, 2012; Sessle 2011b). To prevent persistent pain, decreased facilitation and increased inhibition of movement due to local inhibitory interneuronal activity in M1 mediated by GABA may be necessary to limit muscle activity (Schabrun and Hodges, 2012). In fact, it is suggested that the analgesic effects of M1 stimulation are produced by restoring the defective intracortical inhibition in M1 (Lefaucheur et al., 2006). In the case of the orofacial region, limited movement is apparent in chronic pathophysiological conditions affecting musculoskeletal tissues such as temporomandibular disorders (Kumar and Brennan, 2013).

Some TMS studies in humans have shown no changes in face-M1 excitability following experimental orofacial noxious stimulation induced by capsaicin injection into the periodontal ligament (Zhang et al., 2010) or by hypertonic saline injection into the masseter.
muscle (Romaniello et al., 2000). These findings contrast with those of human studies noted above, and may be explained by methodological differences in EMG recording, or in the case of the Romaniello et al., study, the use of voluntary masseter contraction may have masked a weak inhibitory effect of nociceptive input. However, it is also possible that different types of noxious stimuli applied to different orofacial tissues have varying influences on M1 function. This is consistent with human limb studies showing that experimental noxious stimulation of spinally innervated tissues is associated with different effects on limb-M1 excitability. For example, decreased limb-M1 excitability has been associated with experimental pain induced by capsaicin application to the skin (Farina et al., 2001), hypertonic saline injection to hand muscles (Le Pera et al., 2001; Svensson et al., 2003) and noxious thermal stimulation of the hand (Dube and Mercier, 2011) whereas noxious electrical stimulation of the finger results in increased excitability of M1 regions representing distal hand muscles but decreased excitability of M1 regions representing the proximal arm muscles (Kofler et al., 1998).

### 2.3 Mechanisms underlying changes in Face-M1 excitability and RAD activity and the role of medullary astrocytes

The cellular and molecular mechanisms underlying orofacial pain-induced face-M1 excitability changes are still unclear and the limited relevant literature that is available has focused on mechanisms to explain excitability changes of limb and vibrissal-M1. One of the objectives of the present study was to test whether medullary astrocytes are involved in modulating the MO-induced changes in face-M1 excitability. The following sections discuss some possible mechanisms that may underlie the findings of the present study.

#### 2.3.1. Why astrocytes?

Astrocytes are non-neuronal glial cells found in all regions of the CNS including the brainstem (see Chiang et al., 2011, 2012; Savchenko et al., 2000). Astrocytes function as a syncytium of interconnected cells that are in close spatial relation to neuronal synapses (Pekny and Nilsson, 2005; Verkhratsky and Butt, 2013). Astrocytes have receptors for several mediators (e.g., glutamate, ATP, cytokines), they can release and remove excitatory
and inhibitory neurotransmitters (e.g., glutamate, GABA) to or from the extracellular space, and therefore can sense and modulate neuronal activity and thereby play a crucial role in cortical and subcortical neuroplasticity (for review see Chiang et al., 2011, 2012; Freeman, 2010; Giaume et al., 2010; Haydon and Carmignoto, 2006; Keyser and Pellmar, 1994, Volterra and Meldolesi 2005; Walz, 1989). It has been documented that neurone–astrocyte interactions are key mechanisms underlying central sensitization in acute and chronic pain conditions including orofacial pain (Chiang et al., 2011, 2012; Durham and Garrett, 2010; Ji et al., 2013; Velez-Fort et al., 2012). Astrocytes can be activated in various pathological conditions including acute and chronic pain conditions. Activated astrocytes (i.e., reactive astrocytes) in pain conditions manifest increased GFAP immunoreactivity as well as hypertrophy of their cell bodies and processes (i.e., gliosis) (see Chiang et al., 2011, 2012; Gao and Ji, 2010). It has been documented that MDH central sensitization induced by acute and chronic pulpitis or trigeminal nerve injury is associated with activation of MDH astrocytes (and microglial cells) (Okada-Ogawa et al., 2009; Tsuboi et al., 2011). A process specific to astrocytes is the glutamate/glutamine shuttle that replenishes glutamate in excitatory neurones and the GABA/glutamate/glutamine shuttle that replenishes GABA in inhibitory neurones. The shuttle removes excessive glutamate (or GABA) from the synaptic cleft, thereby collecting glutamate to be converted into glutamine by GS in the astrocyte. Glutamine is then transported back to the presynaptic terminal where it is converted into glutamate in excitatory synapses and into GABA in inhibitory synapses to replenish the supply of neurotransmitters (for review, see Chiang et al., 2011, 2012; Danbolt, 2001; Hertz and Zielke, 2004; Kanamori and Ross, 2006; Verkhratsky and Butt, 2013). Inhibition of the shuttle at inhibitory synapses will impede inhibitory transmission; and inhibition of the shuttle at excitatory synapses will impede excitatory transmission (Hertz and Zielke, 2004; Jiang et al., 2012; Miyake and Kitamura, 1992; Norenberg and Martinez-Hernandez, 1979; Verkhratsky and Butt, 2013). GS is exclusively expressed in astrocytes and MSO is a potent and specific inhibitor of GS (Chiang et al., 2011, 2012; Miyake and Kitamura, 1992; Norenberg and Martinez-Hernandez, 1979). Therefore low doses of MSO as used in the present study have a high specificity in inhibiting astrocyte function without affecting neurones (Amaral et al., 2013; Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979).
2.3.2. Modulation of Vm excitability

Sensory inputs to the VBSNC (including nociceptive inputs to the MDH) can be relayed to the intertrigeminal nucleus, supratrigeminal nucleus and reticular formation through excitatory and inhibitory interneurones. Second-order neurones in these nuclei can then synapse onto motoneurones within the adjacent cranial motor nuclei, including Vm which supplies and regulates the jaw muscles such as the AD (Capra, 1995; Dostrovsky 2006; Paxinos, 2004; Sessle, 2000, 2006, 2009; van Steenberghe and de Laat, 1989). It has been shown that MO application to the molar tooth pulp in anaesthetized rats increases glutamate release in the MDH (Kumar et al., 2012). Electrical stimulation or glutamate micro-injection into the MDH results in increased ipsilateral AD activity (Tsai et al., 1999). Systemic administration of pregabalin, an antagonist of voltage-gated calcium channels at the α2δ subunit, can decrease glutamate release and also attenuate the MO-induced increase in RAD EMG activity (Narita et al., 2012). While it has been shown that application of the astrocyte inhibitor MSO to the MDH can block MDH central sensitization and the increased GFAP (astrocytic)-immunoreactivity within the MDH in the MO tooth pulp application model (Chiang et al., 2007; Varathan et al., 2014) and in trigeminal nerve injury models and chronic pulpitis models (Okada-Ogawa et al., 2009; Tsuboi et al., 2011), it has also been shown that chronic constriction injury of the infraorbital nerve is associated, 3 and 14 days later, with increased activity of Vm motoneurones; subsequent modulation of Vm neuronal activity by application of the astrocyte inhibitor MSO significantly attenuated the injury-induced effects and these MSO effects were reversed by glutamine application to the Vm (Mostafeezur et al., 2014).

The foregoing raises the possibility that an increase in VBSNC neuronal activity induced by MO application to the molar tooth pulp and mediated by glutamatergic transmission can activate glutamate-dependent brainstem excitatory circuits that project to and reflexly activate jaw-opening muscles manifested as an increased RAD EMG activity (Dostrovsky 2000; Paxinos, 2004; Sessle, 2000, 2006, 2009). Furthermore, these changes in Vm neuronal activities may involve the astroglial glutamate/glutamine shuttle in Vm as well as in MDH. However, consistent with Narita et al., (2012) and Sunakawa et al., (1999), we found that the MO-induced increase in RAD EMG activity was transient, suggesting inhibitory mechanisms.
may subsequently be manifested as a result of the MO-evoked afferent input into the CNS. AD reflex activity induced by noxious stimulation of the tooth pulp or other orofacial tissues can be modulated by endogenous descending inhibitory influences through release of chemical mediators such as opioids from the NRM or the PAG (Sessle and Hu, 1981). For example, systemic administration of the opiate antagonist naloxone can rekindle the RAD EMG activity induced by MO application to the molar tooth pulp (Sunakawa et al. 1999). In addition, sustained increased nociceptive inputs induced by hypertonic saline injection into the masseter muscle can variably inhibit or activate Vm motoneurones (Capra et al., 2007). This inhibition may be relayed through the intertrigeminal nucleus, supratrigeminal nucleus or reticular formation, and through inhibitory (e.g., GABA) premotoneurones, inhibit Vm motoneurones (Capra and Dessem, 1992; Kamogawa et al., 1988).

It is possible that these inhibitory influences on Vm motoneurones could also contribute to the decreased face-M1 excitability shown in the present study. Indeed, it has been documented in humans and rabbits that noxious stimulation of limb or jaw muscles, as well as the perioral cutaneous region, results in decreased excitability not only of M1 neurones but also of spinal and brainstem motoneurones (Le Pera et al., 2001; Truini et al., 2006; Westberg et al., 1997). Therefore, although we did not test excitability changes in the Vm, we cannot rule out the possibility that the MO-induced changes in face-M1 excitability observed in the present study also reflect changes in Vm. This view can be further supported by our findings whereby MSO application to the medulla reversed the MO-induced effect on face-M1 excitability. As detailed above, increased input onto inhibitory or excitatory neurones is dependent on the functional integrity of the astrocytic glutamate/glutamine shuttle. It could be argued that blocking the shuttle with MSO at excitatory synapses in MDH or Vm depletes the glutamine supply and decreases glutamate synthesis, resulting in decreased activation of inhibitory premotoneurones (e.g. in MDH) and decreased inhibition (disinhibition) of Vm motoneurones, thereby counteracting the MO-induced decrease in face-M1 excitability. In addition, the inhibitory effects of premotoneurones on Vm motoneurones depend on the GABA-glutamate/glutamine shuttle. Blocking the shuttle with MSO results in a rapid depletion of glutamine, decreased GABA synthesis and subsequently disinhibition (i.e., increase) of motoneuronal activity, thereby counteracting the MO-induced decrease in face-M1 excitability (Mostafeezur et al., 2014). However, changes in the
excitability of Vm motoneurones would be expected to be associated with changes in RAD onset latency (Butovas and Schwarz, 2003; Ridding and Rothwell, 1997; Tehovnik et al., 2006), and the present study did not reveal any significant changes in RAD onset latencies following MO application to the molar tooth pulp. Furthermore, other studies which did measure Vm excitability found that application of the algesic compound capsaicin to the rat vibrissal skin (Kim et al., 2010) was not associated with excitability changes within the vibrissal-M1. Furthermore, application of the algesic chemical glutamate to the rat tongue was associated with a decrease in tongue-M1 excitability but not in the hypoglossal motor nucleus (Adachi et al., 2008). Thus, it is likely that the observed MO-induced decrease in face-M1 excitability reflected, at least in part, changes within face-M1.

In support of face-M1 mechanisms underlying MO-induced decreases in face-M1 excitability, we have recently found that MO application to the molar tooth pulp increases GFAP labelling (reflecting astrocyte activation) in face-M1 (Varathan et al., 2014) and that the MO-induced decreases in face-M1 excitability can also be rapidly (within minutes) reversed by MSO application directly to the face-M1 surface (Awamleh et al., 2014). Therefore, it is suggested that mechanisms involving the astrocytic glutamate/glutamine shuttle within the face-M1 itself are also involved in modulating face-M1 excitability following noxious stimulation of the molar tooth pulp.

2.3.3. Modulation of Face-M1 excitability by nociceptive input

Somatosensory primary afferents from the orofacial tissues (including the teeth) project to the VBSNC where they ascend or descend into the trigeminal spinal tract and give off collateral branches that terminate in different subdivisions of the VBSNC: the main sensory nucleus and the MDH, Vo and Vi of the Vsp (Dallel et al., 1990; Hayashi et al., 1984; Marfut and Turner, 1984; Sessle, 2000, 2006; Takemura et al., 2006). Nevertheless, based on clinical, behavioural, anatomical, electrophysiological and immunohistochemical studies, the MDH has been especially implicated in trigeminal nociceptive mechanisms (Dostrovsky and Sessle, 2007; Dubner et al., 1978; Sessle, 2000). Second-order neurones project either directly or indirectly to the thalamus and other higher brain regions (Dubner et al., 1978; Sessle, 2000; Svensson and Sessle, 2004). From the thalamus, nociceptive information is
relayed to cortical regions including the face-S1 (Dubner et al., 1978; Kenshalo et al., 2000). In humans, brain imaging studies report that acute noxious stimulation of the tooth pulp activates a brain network that includes (but is not limited to) the VBSNC including the MDH ipsilaterally and Vo bilaterally, Vm, motor and sensory thalamic nuclei as well as several regions within the so-called ‘pain network’ that includes the S1 (Ettlin et al., 2009; Jantsch et al., 2005; Lin et al., 2014; Weigelt et al., 2010).

Although the face-M1 is the main cortical region that plays a crucial role in the generation and control of orofacial movements (Avivi-Arber et al., 2011; Sessle, 2001), it is important to note that the face-M1 also receives somatosensory information either directly from the thalamus or indirectly through face-S1 (Chakrabarti and Alloway, 2006; Dubner et al., 1978; Hoffer et al., 2005; Iyengar et al., 2007; Miyashita et al., 1994; Rocco-Donovan et al., 2011). These sensory inputs to the face-M1 provide important feedback and feed-forward information that can assist in (or interfere with) modulating orofacial motor outputs. Human imaging studies confirm that experimental noxious stimulation of the teeth (and other orofacial regions) can also activate face-M1 regions as defined by fMRI and PET (Becerra et al., 1999; de Leeuw et al., 2006; Ettlin et al., 2009; Jantsch et al., 2005; Lin et al., 2014; Nash et al., 2010; Weigelt et al., 2010).

As discussed above, MO application to the rat molar tooth pulp, as well as other orofacial noxious stimuli, induces central sensitization (increased excitability) of nociceptive neurones within the MDH (Chiang et al., 1998, 2002, 2005, 2007, 2010, Xie et al., 2007), as well as the thalamic VPM and MD nuclei (Kaneko et al., 2011; Kaneko et al., 2005; Park et al., 2006; Zhang et al., 2006) and face-S1 (Sessle, 2000; Calford and Tweedale, 1991; Katz et al., 1999; Kawamura et al., 2010; Kupers et al., 2004; Takeda et al. 2010). The MO-induced MDH central sensitization is mediated by glutamatergic transmission, e.g. MO application to the molar tooth pulp in anaesthetized rats increases glutamate release in the MDH (Kumar et al., 2012; Narita et al., 2012) and NMDA receptor blockade in the MDH overcomes the MO-induced central sensitization (Chiang et al., 1998). Application of the astrocyte inhibitor MSO to the MDH can reverse the MDH central sensitization induced by MO application to the molar tooth pulp (Chiang et al., 2007; Chiang et al., 2012). The present study revealed that MO application to the molar tooth pulp induced a rapid (within minutes) decrease in
face-M1 excitability and that MSO application to the medulla reversed the MO-induced decrease in face-M1 excitability. These findings suggest that medullary nociceptive neurones may play a crucial role in nociceptive transmission to the face-M1 (and face-S1) that can modulate face-M1 excitability. However, it is important to note that the MDH may not be the only component of the VBSNC involved. NS neurones also occur in Vo and Vi, and tractotomy or lesioning or blockade of the MDH may have only a limited effect on the nociceptive responses to noxious stimuli (including dental pulp stimulation) within the thalamus and S1 (Dallel et al. 1988, 1989; Raboisson et al., 1989; Sessle, 2000; Sessle and Greenwood, 1976;) while tractotomy or lesioning of the Vo can impede thalamic and S1 neuronal responses to intra and perioral noxious stimuli (Sessle, 2000),

While the present in vivo study suggests that MSO exerted its effect on GS in reactive astrocytes within the medulla, its possible direct effect on medullary neurones needs to be considered since in vitro studies of rat cortical slices have reported that MSO (0.1-0.3 mM) results in the release of glutamate and glutamine and a sustained neuronal activation that can be partially blocked by an antagonist of neuronal NMDA receptors (Albrecht and Norenberg, 1990; Shaw et al., 1999; Zielinska et al., 2004). However, such neuronal activation was unlikely in the present study since the study used a low dose of MSO (0.1 mM) and application of MSO in control animals (which did not receive acute noxious stimulation of the molar tooth pulp) had no effects on baseline RAD thresholds. This indicates that MSO had no effect on non-reactive astrocytes and did not affect basal physiological nociceptive signaling. These findings are also consistent with previous studies in acute (e.g., MO-induced) or chronic orofacial pain models where similar low (0.1mM) concentrations of MSO applied to the MDH were shown to prevent or reverse the MO-induced increased excitability of MDH nociceptive neurones but not affect the basal physiological properties of the MDH nociceptive neurones (Chiang et al., 2007; Tsuboi et al., 2011; Xie et al., 2007).

3. Study limitations and future directions

As listed below, several factors may bear on the interpretation of this study’s findings.

The study was limited by the use of ICMS at only one cortical site and stimulating a limited number of face-M1 neurones at the vicinity of the microelectrode. There are many other
cortical areas and subcortical structures involved in the control of orofacial movement, including the CMA, SMA, basal ganglia, and cerebellum. These areas were not examined in the present study. The use of TMS in humans would have complemented the study as it could have examined broader areas of the cortex, and would have also avoided the craniotomy and exposure of the skull, which may have impacted neuronal and brain health throughout the acute experiments (Shih et al., 2012).

The study results also cannot be extended to female rats. Female rats were not used in this study as estrogen cycling can modulate nociceptive responses (Cairns et al., 2001a, 2007). Therefore, future experiments could examine the effects of acute dental pain on M1 excitability in female rats may e.g. test for possible sex differences.

In addition to glutamate and GABA, astrocytes can also release other neurotransmitters that modulate neuronal excitability, and these transmitters could be the focus of future studies. Astrocytes also release, for example D-serine, which may be responsible for sensitizing neurones as it decreases the threshold for NMDA receptor activation (Guo et al. 2006), and S100B activation, which is elevated in dorsal horn astrocytes in inflammatory and neuropathic pain models (Raghavendra et al., 2004; Tanga et al., 2006). In response to release of glutamate and ATP by neurones, astrocytes can also release glutamate and ATP, leading to further activation of nearby neurones and glial (Milligan and Watkins, 2009).

MSO was applied to the surface of the medulla so we cannot be certain of the exact site of action. The depth of penetration into the caudal medulla by MSO is unknown, so it is possible that only the superficial layers of the caudal medulla were affected, or that deeper nuclei may have also been affected. Future studies could use other techniques such as microinjection and microdialysis to test the exact site of action of MSO.

Future studies could also examine the effects of acute noxious stimulation of the dental pulp on the excitability of the Vm and whether the astrocytic glutamate/glutamine shuttle is indeed involved in modulating the excitability of Vm motoneurones. It would also be informative to carry out recordings of face-M1 and face-S1 neurones following noxious tooth pulp stimulation by MO to measure changes in neuronal excitability as well as the effect of MSO on the neuronal excitability. The use of immunohistochemical labelling and
fluorescence techniques to visualize astrocytes and could complement the
electrophysiological approaches, and be extended to test the possible involvement of
microglial cells in the excitability changes. Single photon imaging of Ca$^{2+}$ waves by use of
Ca$^{2+}$ indicators and optical fibres allows simultaneous visualization of neuronal and
astrocytic networks (Schulz et al., 2012).

4. Clinical implications

Acute dental pain is one of the most common pain conditions and is still a clinical challenge
(Cohen et al. 2009; Lipton et al. 1993; Locker and Grushka, 1987). Without proper
treatment, acute orofacial pain conditions such as dental pain may progress into chronic pain
conditions (Nixdorf et al., 2010; Sessle, 2009b). Despite distinct PNS and CNS mechanisms,
acute and chronic pain conditions are associated with similar behavioural hypersensitivity
and impaired motor functions (Sessle, 2009b; Svensson et al., 2003; Svensson and Graven-
Nielsen, 2001). However, many of the mechanisms underlying the interaction between pain
and impaired motor function, as well as how acute dental pain may contribute to the
development of chronic pain, are unclear.

Therefore, our findings that acute noxious stimulation of the dental pulp may cause
decreased excitability in corticomotor pathways, and that a blockade of nociceptive
transmission by an astrocyte inhibitor at the level of the medulla can restore face-M1
excitability, suggest that the medulla plays a crucial role in transmitting sensory information
to the face-M1 (and face-S1), that nociceptive afferent inputs can decrease face-M1
excitability, and that medullary astrocytes are involved in these effects. These findings
suggest that medullary astrocytes can be therapeutic targets for treatment and prevention of
acute pain and associated motor dysfunction, and for the prevention of acute pain progression
into chronic pain.

5. Conclusions

This study has demonstrated for the first time that application of the inflammatory irritant
MO to the rat right maxillary molar tooth pulp induced neuroplastic changes reflected in
decreased excitability of contralateral face-M1. This decreased face-M1 excitability was
manifested as increased ICMS threshold intensities required to evoke EMG activity in the RAD muscle. This study also provided novel findings that application to the caudal medulla of an inhibitor (MSO) of the astrocyte enzyme GS could reverse the MO-induced effects. In contrast, neither MO application to the molar tooth pulp nor MSO application to the medulla had any effect on the onset latencies of the ICMS-evoked RAD EMG activities. Therefore, these novel findings suggest that medullary nociceptive neurones may play a crucial role in transmitting nociceptive information to the face-M1 that can modulate face-M1 excitability and that mechanisms involving medullary astrocytes may contribute. These findings may have clinical implications as they provide new insights into the possible mechanisms by which acute pain in humans is associated with limited jaw movements and the finding of the involvement of astrocytes may offer a novel therapeutic target for the prevention and treatment of acute dental pain and impaired motor function.
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